RETURN TO H. W. HOWARD

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
VOLUME 57, ART. 6   PAGES 615–962

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ROY WALDO MINER

NUTRITIONAL FACTORS AND LIVER DISEASES

BY

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1817

NEW YORK
PUBLISHED BY THE ACADEMY
May 10 1954

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ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

VOLUME 57, ART. 6, PAGES 615–962

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Editor
ROY WALDO MINER

NUTRITIONAL FACTORS AND LIVER DISEASES*

Consulting Editor and Conference Chairman: KLAUS SCHWARZ

RECEIVED
MAY 28 1954
H. W. H.

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* This series of papers is the result of a Conference on *Nutritional Factors and Liver Diseases* held by the Section of Biology of The New York Academy of Sciences, February 13 and 14, 1953.

Publication has been aided by a grant-in-aid from the Wenner-Gren Foundation for Anthropological Research.

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INTRODUCTION: LIVER NECROSIS VERSUS FATTY LIVER AND CIRRHOSIS

By Klaus Schwarz

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> Part of our knowledge we obtain direct and part by argument.
> —JOHN MAYNARD KEYNES, *A Treatise on Probability.*

It is, of course, a great pleasure to write the introduction to this monograph based on the Conference on "Nutritional Factors and Liver Diseases," which it has been my privilege to plan and to organize. The concepts and intentions which have guided this endeavor can be outlined as follows. Within the limitations of a two-day meeting and the following collection of papers presented, an attempt has been made to assemble a very broad spectrum of our present knowledge and of all current trends in research on nutritional liver disease, to put before you a real "symposium." What appears more important, however, is that an effort has been made to put our various pieces of knowledge together in such a way as to form a clearer picture out of the rather chaotic development which this field has undergone. This may permit us to visualize and to understand possible relations between heretofore isolated areas; to clarify, at least in part, some of the complex aspects of this field, which seemingly have been lacking integration so far.

To achieve this purpose, several heterogeneous fields had to be brought together and connected in a coordinated fashion, the two most important ones being biochemical research in experimental animals on one side and clinical and pathological research on humans suffering from dietary liver disease on the other. Workers not only from various scientific fields but also from different continents had to be brought together since the investigation of experimental dietary liver diseases has advanced most in North America and in Europe, whereas work in human nutritional liver injury has by necessity been carried out more intensely in Latin America, Asia, and especially in Africa, where Cecily Williams made her classical observations on kwashiorkor in 1933.[1] Our appreciation is extended to all the workers from abroad who have contributed so effectively to the meeting and to this volume.

The guiding consideration in the arrangement of the following material is based on the results of animal experimentation. It is quite certain that, in experimental animals, two completely different deficiency syndromes can be distinguished clearly. These are fatty liver and cirrhosis on the one hand, and acute (massive) liver necrosis, preferably called necrotic liver degeneration,* on the other. These two disease entities can be produced separately and even occupy a mutually antagonistic position in response to various dietary factors which are protective (see below). According to this clear-cut separation, the conference program and this monograph are separated into two main parts, one being devoted to fatty liver and cirrhotic diseases and the other to necrotic liver injuries. Each main part, in turn, was scheduled originally to present, first, the results of animal experimentation and, secondly, the clinical investigations of corresponding nutritional liver injuries in the human. A certain imbalance is evident, however, since experience with human fatty liver and cirrhosis due to malnutrition is abundant indeed, whereas knowledge of human necrotic liver disease due to malnutrition is quite inadequate as yet.

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* "Degeneration" is defined as progressive deterioration of tissue in which its vitality is diminished, whereas "necrosis" means death of a circumscribed portion of tissue. "Dietary necrotic liver degeneration" is applied to define a deficiency disease which in previous publications has been called "hemorrhage," "hemorrhage and necrosis" or "liver degeneration." The defect is characterized by a degenerative change which eventually leads to acute extensive liver necrosis and death.

Nutritional Liver Disease in Humans (*Kwashiorkor*)

It has become quite clear that nutritional disease of the liver occurs very frequently in small children and also in adults, especially in tropical and in subtropical areas, as part of the so-called kwashiorkor syndrome. The disease constitutes a world-wide problem. It is one of the most important dietary deficiencies in mankind today. This is not yet sufficiently realized in some countries, partly due to indifference and partly due to lack of information and of communication. It is often fatal and poses difficult and challenging problems in the field of clinical medicine and in nutrition research. The disease is not really new. It has been described under a great variety of names, for example as kwashiorkor,[2] culebrilla, syndrome policarential, malignant malnutrition, distrophie de farineuse, and as far back as 1906 as Mehlnährschaden.[3] The African term kwashiorkor has been officially accepted by the Joint Expert Committee on Nutrition of the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) of the United Nations.

In the liver, the major features of kwashiorkor are fatty infiltration and cirrhosis. These changes are apparently intimately related, if not identical, to fatty infiltration and cirrhosis in areas with temperate climates, particularly among alcoholics. For this reason, the nutritional theories of the etiology of alcoholism, as well as the dietary aspects of the origin and the treatment of alcoholic fatty liver and cirrhosis have been considered as pertaining to the general theme of this volume. The dietary factors which protect against human fatty liver and cirrhosis have not been elucidated as yet.

Experimental Nutritional Liver Disease

In experimental animals, dietary liver diseases (*i.e.*, liver injuries produced by deficient diets) have been known since approximately 1930. During the first decade of research in this field attention was focussed on fatty infiltration and liver cirrhosis.[4, 5] The significance of an article by Weichselbaum in 1935,[6] describing "hemorrhages throughout the liver" and "clear signs of icterus" which were produced by a cystine deficient diet was not realized until much later. When du Vigneaud and his associates produced liver necrosis in rats on amino acid diets in 1939, they devoted only one paragraph in small print to the description of the phenomenon.[7] Between 1939 and 1946 difficulties were encountered in the differentiation of these two types of liver diseases, and the resolution of these difficulties was seriously delayed by the international scientific blackout during World War II.

In doing experimental research with purified diets, we have, allegedly, the advantage of being able to deal with "pure" forms, with just one variable at a time, whereas the clinician deals with more complex situations. However, it is characteristic for both experimental fatty liver/cirrhosis and liver necrosis, that they are the result of rather complicated dietary deficiency situations. Both of these injuries are not the result of single dietary defects, but are caused by the simultaneous lack of several essential nutrients. In both diseases some of the major dietary factors have been identified, but hitherto unknown protective factors are also involved. Hence, it is understandable that research workers have not always succeeded in producing pure forms of these diseases. In 1939, papers appeared describing the occurrence of both liver necrosis and fatty infiltration/cirrhosis in the same animals. These had been kept for extended periods on low protein rations which were deficient in quite a variety of essential dietary components. Thus, both types of nutritional liver disease were produced simultaneously, leading some workers to think that those lesions constituted a single deficiency syndrome. This somewhat handicapped progress in the clarification of the factors involved. In 1942 it was clearly established by Daft, Sebrell, and Lillie[8] that the two diseases were separate entities. This, however, did not prevent the subsequent publication of occasional papers which failed to discriminate between the fatty liver/cirrhosis syndrome and dietary necrotic liver degeneration. In Europe, during this same period, several dietary methods were found which produced necrotic liver degeneration at high incidence and in a reproducible manner.[9, 10, 11] Signs of fatty infiltration/cirrhosis were not seen since the rations were supplemented with choline. The necrotic deficiency disease was found to be prevented by cystine[9] and in 1944 vitamin E was disclosed to be highly protective.[12] More recently a third, not yet identified factor, Factor 3, has been shown to be involved.[13] Each one of these three substances alone affords protection.

TABLE 1

	LIVER NECROSIS	FATTY LIVER CIRRHOSIS
Disease process	degenerative metabolic change of the liver parenchyma leading to sudden, acute attack of massive necrosis	fatty metamorphosis of liver with slowly developing fibrosis cirrhosis
	No fatty infiltration *No* fibrosis	*No* (massive) necrosis
Residual signs	postnecrotic scarring	cirrhosis
Protective factors:		
Cystine*	protects	*enhances*
Vitamin E	protects	without effect
Factor 3	protects	not known
Choline	*enhances*	protects
Betaine	*enhances*	protects
Methyl group precursors	*enhances*	protects
Vitamin B_{12}	*enhances*	protects
Folic acid/citrovorum factor	*enhances*	protects

* Methionine has been shown to be protective against fatty liver and cirrhosis; it contains a labile methyl group. It is also slightly protective in dietary necrotic liver degeneration, since it is partly transformed into cystine in intermediary metabolism.[14]

Thus it became obvious that the group of dietary factors which prevents necrotic liver degeneration is completely different from the group of factors which protects against fatty liver/cirrhosis. Furthermore it could be shown that substances which prevent the development of fatty liver and cirrhosis, such as choline, betaine, precursors of one carbon units, vitamin B_{12} and folic acid/citrovorum factor enhance the development of the necrotic liver injury.[14] On the other hand, cystine, which protects against necrotic degeneration, accentuates the fatty liver/cirrhosis syndrome. The situation is summarized in TABLE 1.

Regarding the Relationship of Experimental and Human Nutritional Liver Disease

It is evident from this monograph that the various phases of experimental and of human nutritional liver disease can be put together in such a way that a great number of parallels become visible. This is particularly true for the fatty liver/cirrhosis diseases. However, there are also obvious discrepancies. As to necrotic liver injury, the situation is much less clear; the occurrence of nutritionally induced necrotic disorders in the human remains to be established. I want to state explicitly that the obvious parallelisms do not necessarily indicate true identity of biochemical and pathological events involved.

Much has been achieved in experimental nutritional liver disease even though there are still many important questions and problems which have not been clarified. Knowledge of human nutritional disorders of the liver, by comparison, is much less advanced. The more or less close parallelisms between human liver disease and the two main types of experimental dietary liver injury have of course led to conjecture and to premature discussion of the question of how much human liver disease is induced nutritionally and what might be done with liver-protecting factors in therapy. As long as we do not know all the dietary factors necessary to protect, these questions cannot be answered. Early hopes, for example, that choline, which is protective against fatty liver and cirrhosis in the rat, would prevent or cure fatty liver and cirrhosis in the human have not been fulfilled. This has led some to discredit the nutritional line of approach. Since then we have learned that numerous factors other than choline are involved in methyl group and in one carbon unit metabolism, such as, for example, vitamin B_{12} and the folic acid/citrovorum factor group. Between different species or even between different strains of the same kind, differences in the relative importance of these various essentials are naturally bound to occur, and we should not expect the human to respond in all details exactly as the rat does.

The conclusion which can be drawn from this example is that much more careful studies are necessary before the true relationship between experimental and human dietary liver injury can be ascertained. Such relationship is bound to exist, but at present we can not recognize it precisely. It is hoped that a full exchange of knowledge and of opinions—often controversial—will be helpful to both the laboratory investigator and the clinician. This exchange is what has been attempted here.

Among biochemists, pathologists, and doctors there are differences of approach and of thinking, with resulting possibilities of misunderstanding. There are also pitfalls in terminology and in semantics. Bringing these groups together constituted something of an experiment in itself, one which appeared well worth trying.

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Part I. Fatty Liver and Cirrhosis

EXPERIMENTAL DIFFERENTIATION BETWEEN LIVER NECROSIS AND LIVER CIRRHOSIS AND SOME DIETARY FACTORS AFFECTING THEIR DEVELOPMENT

By Floyd S. Daft

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It is now generally recognized that dietary liver cirrhosis and dietary liver necrosis are separate and distinct entities, both etiologically and morphologically. That is not to say that death of cells does not occur as part of the cirrhotic process or that fibrosis may not follow necrosis. Most observers of experimental dietary cirrhosis have reported the presence of scattered necrotic liver cells. I believe that none would contend, however, that massive necrosis is part of this process. Extensive scarring following massive necrosis has been observed also in several laboratories. We at the National Institutes of Health, however, have never observed diffuse fibrosis following dietary necrosis and the same appears to be true of most other groups of investigators. Doctor Hoffbauer will discuss this matter at some length in a later paper. It suffices to say here that there are two distinct processes, experimental dietary cirrhosis and experimental dietary necrosis, which may occur either separately or in the same liver in rats which have received certain deficient diets.

At this time, I should like to review briefly the historical background of this concept and present some additional data in its support. In addition, I should like to discuss a few experiments carried out in our laboratory on the influence of dietary ingredients, other than those previously implicated, on the development, particularly, of fatty livers and etiologically related lesions.

The first clear differentiation between dietary liver cirrhosis and dietary liver necrosis was made in 1942 by Daft, Sebrell, and Lillie.[1, 2] In our first publication on this subject, in *Federation Proceedings*,[1] we stated, "Under the conditions of our experiments, choline prevents the cirrhosis, cystine prevents the hemorrhagic necrosis, and methionine prevents both the cirrhosis and the hemorrhagic necrosis. It appears, therefore, that the cirrhosis and the hemorrhagic necrosis are separate and distinct pathological entities." In our more complete report, published a few months later in the *Proceedings of the Society for Experimental Biology and Medicine*,[2] our conclusions were worded somewhat more conservatively, but the reasons for our belief that we were dealing with two distinct deficiency syndromes were clearly stated. The evidence on which our conclusions were based consisted of morphological observations and the results of dietary experiments. In regard to the morphological findings, we stated "Some rats on this same diet developed hemorrhage and necrosis of the liver. This necrosis appears to be identical with that seen by György and Goldblatt.[3] It is primarily coagulative in type and centrolobular in location and is nearly always accompanied or even replaced by hemorrhage of quite variable extent in the necrotic areas. Sometimes only small periportal islets

TABLE 1

BASAL DIETS EMPLOYED IN CIRRHOSIS AND NECROSIS EXPERIMENTS

	Basal diet No. 1	Basal diet No. 2	Other basal diets
Casein	4.0%*	4.0*	4.0** to 18.0**
Cystine	—	0.04%	—
Salt mixture†	4.0	4.0	4.0
Cod-liver oil	2.0	2.0	2.0
Vegetable oil‡	3.0	3.0	3.0
Corn starch	87.0	86.96	—
Sucrose	—	—	to 100

* Leached.
** Leached and hot alcohol extracted.
† Osborne and Mendel.
‡ Wesson oil.

of surviving liver cells remain. When this picture and the hepatic cirrhosis described by us[4] occur together, they appear to be essentially unrelated to each other, the hemorrhage and necrosis being superimposed on almost any phase in the development of the cirrhosis. Rarely is there any evidence of marginal organization about the hemorrhagic necrosis, and usually the hemorrhage is so fresh that no hemosiderin is demonstrable."

The basal diet used in these early experiments was No. 1 and is shown in TABLE 1. It consisted of leached casein 4 per cent, Osborne and Mendel salt mixture 4 per cent, cod-liver oil 2 per cent, Wesson oil 3 per cent, and corn starch 87 per cent. A vitamin supplement, consisting of 100 micrograms of thiamine, 50 micrograms of riboflavin, 50 micrograms of calcium pantothenate, 20 micrograms of pyridoxine, and 1 milligram of niacin was given daily. This diet was obviously deficient, therefore, in protein and in choline and was marginal in vitamin E. The most severe amino-acid deficiencies were probably of the sulfur-containing cystine and methionine, although threonine was also severely limiting for growth. At the time these experiments (summarized in TABLE 2) were carried out, we were greatly interested in the effect of alcohol consumption of the exacerbation of the cirrhotic process. For this reason, this particular group of animals received, from the time they were placed on the experimental diet at weaning, 20 per cent alcohol as the sole source of fluid. The effect of methionine, you will note, is compared in the table in each case with that of a somewhat lower percentage of cystine. This represents an approximate matching of molecular equivalents. As was pointed out in our original publications, the two types of hepatic lesions are clearly differentiated by these dietary data. With increasing levels of added cystine, the incidence of cirrhosis increased while the incidence of necrosis (except at the 0.04 per cent level) decreased. Choline chloride (20 mgm. per rat per day) protected against cirrhosis but not against necrosis. Methionine, being a precursor of both cystine and choline, protected against both types of lesions when given in sufficient amounts. The only exceptions to the general rule were three animals receiving choline and cystine. Two of these died after 201 and 245 days, respectively, on experiment and showed slight liver cirrhosis. The third died after 296 days on experiment and showed hemorrhage and

TABLE 2

DIFFERENTIATION OF DIETARY DEFICIENCIES IN EXPERIMENTAL CIRRHOSIS AND NECROSIS. (20% ALCOHOL AD LIB.)

	No. of rats	Cirr. %	Nec. %	Neg. %
Basal diet No. 1	16	19	37	62
Basal diet No. 1 + Cystine 0.04%	17	53	59	24
Basal diet No. 1 + Cystine 0.04% and Choline*	10	0	60	40
Basal diet No. 1 + Meth. 0.05%	27	19	41	52
Basal diet No. 1 + Cystine 0.08%	9	100	22	0
Basal diet No. 1 + Cystine 0.16%	12	92	0	8
Basal diet No. 1 + Meth. 0.20%	4	0	0	100
Basal diet No. 1 + Cystine 0.50%	94	90	0	10
Basal diet No. 1 + Cystine 0.50% and Choline*	15	13	7	80
Basal diet No. 1 + Meth. 0.70%	10	0	0	100

* 20 mg. choline chloride per rat per day.

necrosis. It is worthy of note that 0.05 per cent of added methionine was insufficient to protect against either cirrhosis or necrosis and that 0.04 per cent of added cystine appeared, if anything, to increase the incidence of necrosis as well as cirrhosis.

Prior to this work of ours in 1942, several important researches in the field of dietary liver damage had been carried out. Weichselbaum,[5] in 1935, had reported coma and death in rats deficient in cystine and methionine and had stated that post mortem examination showed definite hemorrhages throughout the liver. No histological examinations appear to have been made, but an excellent clinical description of the deficiency state was included in this report. György and Goldblatt,[3] in 1939, published a detailed description of the lesion, and in the same year du Vigneaud and co-workers[6] described hemorrhage and cell damage in the livers of rats which had received diets deficient in the sulfur-containing amino acids. In none of these early experiments, quite naturally, can the amount of choline which the rats received, either from the diet or from vitamin supplements, be stated with any great degree of certainty. From the descriptions of the lesions, however, it appears that no hepatic cirrhosis was seen by Weichselbaum or by du Vigneaud and associates. György and Goldblatt, however, stated that cirrhosis occurred with necrosis in their animals.

Almost simultaneously with the publication of our differentiation between dietary cirrhosis and dietary necrosis in 1942, György and Goldblatt reported data very similar to those which we presented with, however, a different interpretation of the experimental results. The following year, Hock and Fink[7] described liver necrosis in rats given a diet in which yeast, in rather large amounts, served as the sole source of protein. These investigators reported also that the inclusion of cystine in this yeast diet prevented the development of the hepatic necrosis. This finding furnished new evidence that dietary necrosis is a separate and distinct entity from dietary cirrhosis.

A most important finding, not only to the differentiation of these two types of lesions but also to the whole understanding of the influence of dietary factors or their lack on the integrity of the liver, came in 1944. At this time it was reported by Schwarz[8] that the dietary liver necrosis which he had observed in many hundreds of animals over the previous several years could be prevented by the administration of vitamin E. His method of developing liver necrosis in rats was to give them a diet containing 15 per cent of alkali-treated casein as the sole source of protein. There was a high incidence of liver necrosis with no liver cirrhosis. The prevention of this lesion by vitamin E together with the more recent report by Schwarz[9] in 1951 of the existence of a third factor effective against dietary liver necrosis, strengthens still further the case for the etiological and morphological separation of the two types of liver lesions.

By 1944, then, dietary cirrhosis and dietary necrosis had been fully described, a clear differentiation of the two types of lesions had been made, both on morphological grounds and on the basis of their separation by dietary means, and diets had been described on which necrosis alone developed. In addition, it was in that year that the identification of a second factor against dietary necrosis, vitamin E, was announced.

Before leaving the historical part of this presentation, however, perhaps one additional fact should be recorded. Himsworth and Glynn,[10] in 1944 published the first of their series of papers in which they corroborated many of the findings of earlier investigators. In the same year, Glynn and Himsworth[11] described in some detail an extensive postnecrotic scarring which followed massive necrosis in some of their rats. This lesion had not been observed previously and indeed cannot easily be obtained with the diets which were in use in most laboratories at that time. It is particularly important to make this clear since Himsworth and associates appear not to have claimed the priority which is their due for this interesting observation. They appear, instead, to have credited various American pathologists with including it in the earlier descriptions of cirrhosis.

Tables 3 to 5 show the results of some further studies in our laboratory. They were carried out in collaboration with Doctors Kenneth Endicott and Ralph Lillie who made all of the histological examinations. The basal diet and the vitamin supplement used in these experiments were the same as those

Table 3

Differentiation of Dietary Deficiencies in Experimental Liver Cirrhosis and Necrosis (Water ad Lib.)

Basal diet No. 2		+ Choline*		+ Cystine†		+ Choline* + Cystine†	
Cirr.	—	—	—	Cirr.	—	—	—
Cirr.	—	—	Nec.	Cirr.	—	—	—
—	Nec.	—	Nec.	Cirr.	—	—	—
Cirr.	—	—	—	Cirr.	—	—	—
Cirr.	—	—	—	—	—	—	—
—	Nec.	—	—	Cirr.	—	—	—
Cirr.	Nec.	—	Nec.	Cirr.	—	—	—
Cirr.	Nec.	—	Nec.	Cirr.	—	—	—
—	—	—	—	Cirr.	—	—	—
—	Nec.	—	—	—	—	—	—

* 20 mg. choline chloride per rat per day.
† Additional 0.12% cystine in diet.

Table 4

Differentiation of Dietary Deficiencies in Experimental Liver Cirrhosis and Necrosis (20% Alcohol ad Lib.)

Basal diet No. 2		+ Choline**		+ Cystine†		+ Choline** + Cystine†	
Cirr.	—	—	Nec.	Cirr.	—	—	—
Cirr.	Nec.	—	—‡	Cirr.	—	—	—
Cirr.	—	—	Nec.	Cirr.	—	—	—
—	Nec.	—	Nec.	Cirr.	—	—	—
—	—	—	Nec.	Cirr.	—	—	Nec.
Cirr.	Nec.	—	Nec.	Cirr.	—	—	—
Cirr.	Nec.	—	Nec.	Cirr.	—	—	—
—	Nec.	—	Nec.	Cirr.	Nec.	—	Nec.
—	—	—	—‡	Cirr.	—	—	Nec.
—	Nec.	—	Nec.	Cirr.	—	—	—

* 20% alcohol *ad lib.*
** 20 mg. choline chloride per rat per day.
† Additional 0.12% cystine in diet.
‡ Died after 3 and 15 days respectively.

in earlier studies except that 0.04 per cent of cystine replaced an equivalent amount of corn starch in the diet (basal diet No. 2). It appeared from the previous small experiment (TABLE 2) that this level of cystine might lead to an increased incidence of hepatic lesions. The findings for individual animals are presented in TABLES 3 and 4 in order to show that an occasional rat had neither type of lesion while several had both types. The results in TABLE 3 clearly portray the effect of choline in suppressing cirrhosis but not necrosis, the effect of cystine, even this additional 0.12 per cent, in preventing necrosis while increasing or at least not decreasing the incidence of cirrhosis, and the effect of the combination in preventing both types of lesion. TABLE 4 gives the results obtained with a similar group of animals which received approximately 20 per cent of alcohol as the sole source of fluid. The results are very similar to those in TABLE 3, except for the higher incidence of lesions in some groups and particularly the fact that four animals receiving the higher level of cystine, three of which were receiving choline as well, died with liver necrosis. The possibility that the ingestion of alcohol may increase the incidence of this lesion probably deserves further study. It is interesting that the animals receiving choline had a slightly higher incidence of necrosis than those not receiving this supplement. The only animals receiving the basal diet plus choline which did not develop this lesion were two animals which died after 5 and 15 days, respectively, on experiment. The four animals on the basal diet which did not develop the lesion died after 42, 81, 98, and 110 days, respectively, on the experiment. TABLE 5 summarizes the results in TABLES 3 and 4 and permits a comparison of the findings with water and 20 per cent alcohol, respectively, as the sources of fluid.

All of these experiments were carried out with casein, at a dietary level of 4 per cent, as the sole source of protein. In our experiments, as in those of other investigators, the development of dietary liver cirrhosis and the development of dietary liver necrosis are both dependent to a large extent on the level of protein in the diet. The reason for this is clear since casein, as usually

TABLE 5
SUMMARY OF TABLES 3 AND 4

	No. of rats	Cirr.	Nec.	Neg.
Basal diet No. 2 (water)	10	6	5	1
Basal diet No. 2 (alcohol)	10	5	6	2
Basal + Choline* (water)	10	0	4	6
Basal + Choline* (alcohol)	10	0	8	2
Basal + Cystine† (water)	10	7	0	3
Basal + Cystine† (alcohol)	10	10	1	0
Basal + Choline* and Cystine† (water)	10	0	0	10
Basal + Choline* and Cystine† (alcohol)	10	0	3	7

* 20 mg. choline chloride per rat per day.
† Additional 0.12% cystine in diet.

TABLE 6
EFFECT OF PROTEIN LEVEL

Casein	No. of rats	Fatal kidney lesions %	Cirr. %*	Nec. %*	Neg. %*
4%	10	0	40	20	50
6%	10	0	40	50	20
8%	12	8	82	55	0
10%	12	42	100	43	0
12%	21	24	44	25	56
14%	11	27	25	0	75
16%	11	36	0	0	100
18%	11	18	0	0	100

* Of those not dying of kidney lesions.

prepared, even so-called "vitamin-free" casein, has been shown by Schwarz to contain Factor 3, which prevents liver necrosis, and of course contains also cystine and methionine. Both of these amino acids, as has been indicated, help to prevent the necrosis while methionine is very active in the prevention of cirrhosis. TABLE 6 gives some of our data on this point. In this group of experiments, the diets which were used contained leached and hot alcohol-extracted casein, 4 to 18 per cent, salt mixture 4 per cent, cod-liver oil 2 per cent, Wesson oil 3 per cent, and sucrose to make up the balance. Vitamins were given as in the earlier experiments. Water was the source of fluid. A number of these animals died from the acute kidney lesions first described by Griffith and Wade.[12] It will be noted that in this small group of experiments the highest incidence of fatal kidney lesions occurred on 10 per cent to 16 per cent casein, the highest incidence of cirrhosis on 8 per cent or 10 per cent casein, and the highest incidence of liver necrosis on 6 per cent to 10 per cent casein. In this series, no fatal kidney lesions occurred on levels of casein below 8 per cent, no cirrhosis on levels of casein above 14 per cent, and no necrosis on levels of casein above 12 per cent. In passing, it might be noted that the suggestion has occurred frequently in the literature that rapid growth is essential for the development of kidney lesions. Rapidity of growth is certainly a factor, but we have had no trouble in obtaining fatalities from this lesion routinely on

TABLE 7

CIRRHOSIS "SCORE" AS AFFECTED BY RIBOFLAVIN INTAKE

Riboflavin level (micrograms per rat perday)	Experiment number				
	1	2	3	4	5
5	2.0	2.3	2.3	2.0	1.6
50	1.2	1.4	1.6	1.3	1.1

Exp. 1–3. No choline added to diet.
Exp. 4–5. 25 mg. choline chloride/100 g. diet.

diets too low in protein to permit growth, by including cystine, high levels of fat, and cholesterol in the diet.

I should like to pass now to some studies on the effect of a low riboflavin intake on the development of liver cirrhosis in rats which were carried out in collaboration with Doctor George Fite. These studies were prompted in part by the observations concerning "yellow liver" in dogs by Sebrell and Onstott[13] in 1938. In the light of information accumulated in large part since their experiments were carried out, it appeared possible that the condition they described might have developed as a result of combined riboflavin and choline deficiencies. There were also the numerous clinical studies using diets similar to the high-protein, high-vitamin diet of Patek and Post[14] which seem to indicate that nutritional deficiencies other than that of choline are of importance in clinical liver cirrhosis. In addition, there is the well-known protection of the liver from certain toxic materials such as butter yellow[15] by an increased intake of riboflavin. The diets used in these experiments contained so-called "vitamin-free" casein (G.B.I.) 10 per cent, a modified Osborne and Mendel salt mixture* 4 per cent, cod-liver oil 2 per cent, Wesson oil 3 per cent, and sucrose 81 per cent. The diet of groups 4 and 5 in TABLE 7 contained also 25 mgm. per cent of choline chloride. Two vitamin supplementations were used, the one which I have previously described and another which differed only in that the riboflavin level was reduced from 50 mg. to 5 mg. per rat per day. At weaning, Osborne and Mendel rats were divided equally on a litter and sex basis and maintained under the experimental conditions described for one year unless death intervened. The seven groups in the table represent a total of 176 rats. Following death or sacrifice of the animals, histological examinations were made by Doctor Fite. Each liver was given a score; 0, if no cirrhosis could be observed, or 1, 2, or 3, if cirrhosis was observed, the number assigned depending on the severity. These figures were then averaged for the group. The higher figures in the table, therefore, represent the greater average incidence and severity of cirrhosis. It appears that the animals which received the 5 mgm. level of riboflavin in all experiments had a greater degree of cirrhosis than their litter mates which received 50 mgm. of this vitamin daily. The probability of such an outcome due to chance alone in 5 consecutive experiments is 1 in 32. The result is significant at the .03 level. On the

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* Prepared according to the directions of Osborne and Mendel, except that the sodium fluoride is reduced to 1 per cent of their level and 0.313 gm. of $CuSO_4 \cdot 5H_2O$ (equivalent to 0.2 g. anhydrous $CuSO_4$) is added.

TABLE 8

EFFECT OF COMPOSITION OF FAT COMPONENTS OF DIET ON DEVELOPMENT OF KIDNEY LESIONS

	No. of rats	Acute lesions	Healing or scars	Neg.
		%	%	%
Stearic acid	67	52	24	24
Palmitic acid	49	39	20	41
Oleic acid	48	0	17	83
Soybean oil	8	0	0	100
Soybean flakes	8	37	25	37

basis of these results we should like to propose that the level of riboflavin in a diet, not completely adequate in other factors, may influence the development of liver cirrhosis.

Finally, I should like to present the results of some experiments on the effect of the composition of the fat component of the diet on the development of fatty livers and on the development of kidney hemorrhage and necrosis. These studies were carried out in collaboration with Doctor Kenneth Endicott who made all of the histological examinations. The general plan of the studies was for weanling Wistar or Sprague-Dawley rats to be divided according to sex and litter and given one of three experimental diets. These diets contained casein, which had been leached and extracted with both cold and hot alcohol 4 per cent, modified Osborne and Mendel salt mixture 4 per cent, cystine 0.5 per cent, sucrose 81.5 per cent, and palmitic, stearic, or oleic acid 10 per cent. In a small experiment covered by the last two lines in TABLE 8, soybean oil, or soybean flakes which were prepared by the hydrogenation of this particular lot of soybean oil, was substituted at the same level for the 10 per cent of fatty acid. Both sexes of rats were used, the ratio in each group being roughly three males to one female. In the case of 36 animals, 12 from each of the first three groups, the experiment was terminated after 10 to 14 days and the livers were analyzed for total fat. In the case of 35 additional animals in these three groups, the experiment was terminated by sacrifice of the animals at 16 to 21 days. The remaining 93 rats divided between the three groups were kept on these three diets until their deaths which occurred after experimental periods ranging from 11 days to 216 days. As may be seen from the table, the incidence of acute kidney lesions ranged from 0 per cent for those receiving oleic acid to 39 per cent for those receiving palmitic acid and 52 per cent for those receiving stearic acid. Were we to exclude the animals which were sacrificed, the results would be even more striking. None of 22 animals on oleic acid, 52 per cent of 29 animals on palmitic acid, and 69 per cent of 42 animals on stearic acid showed acute lesions when the experiments were permitted to proceed to the death of the animal. It will be noted further that the results of a small experiment, carried out with unhydrogenated soybean oil and with the same lot of oil hydrogenated to a form known as soybean flakes, support the idea that the degree of saturation of the fat or fatty acid which makes up the fat portion of the diet influences very greatly the incidence of this fatal acute

TABLE 9

LIVER FAT AS AFFECTED BY NATURE OF FATTY ACID INGESTED

	No. of rats	Fat	
		%	Range
Palmitic	9	25.4	(12.0–38.0)
Stearic	9	18.3	(13.4–27.9)
Oleic	9	16.2	(11.2–25.0)

choline-deficiency syndrome. TABLE 9 gives the fat analyses of the livers of nine groups of litter mates. There appears to be little difference in the fat content of the livers of the rats receiving oleic or stearic acid. The level of liver fat of the rats which received palmitic acid appears to have been somewhat higher.

An insufficient number of the 93 animals survived the acute kidney lesions in this series of experiments to permit us to draw any definite conclusions as to the incidence and severity of cirrhosis. I shall merely mention that of the animals thus surviving, one out of 13 or 8 per cent on stearic acid, three of 14 or 21 per cent on palmitic acid, and six of 22 or 27 per cent on oleic acid were found to have some degree of cirrhosis. The oleic acid animals had not only the highest incidence of cirrhosis but also showed the more severe lesions. These fatty acids, as might have been expected from our earlier work,[16] appeared to be very poor precursors of ceroid, since even in those livers which were cirrhotic there was either none of this pigment or only a trace. One rat receiving palmitic acid and one receiving oleic acid showed massive liver necrosis.

In conclusion, we should like to suggest on the basis of these results that the nature of the fatty acids in the fat component of the diet deserves consideration in studies of fatty livers, acute choline deficiency, and hepatic cirrhosis. It will be of interest to determine whether or not the nature of the fat component of the diet is of importance in the development of liver necrosis as well.

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Discussion of the Paper

DOCTOR PAUL GYÖRGY (*School of Medicine, University of Pennsylvania, Philadelphia, Pa.*): The distinction of acute hepatic necrosis from chronic fibrosis, as first proposed by Doctor Daft and Doctor Sebrell, has proved to be a very useful and fruitful concept. In experimental hepatic injury in rats, the etiologic dietary factors for necrosis and cirrhosis appear to be definitely different. In our own laboratory, we have observed independently of Doctor Daft and Doctor Sebrell the beneficial effect of cystine and methionine in experimental necrosis of the liver and the injurious effect of cystine regarding cirrhosis. Nevertheless, we have abstained from a clear-cut separation of necrosis from cirrhosis for two reasons: (1) In toxic hepatic injury, the hepatotoxin in large doses may produce necrosis and, in small repeated doses, typical Laennec's cirrhosis; and (2) Cystine in amounts not much in excess of the therapeutic dose-range will not only prevent but produce hepatic necrosis.

Later, it has been found, first by Schwarz, then independently in our laboratory, that hepatic necrosis is preventable not only by sulfur-containing amino-acids but also by vitamin E. This raised the question whether necrosis is a simple or conditioned deficiency.

THE SEQUENCE OF PATHOLOGIC EVENTS IN THE DEVELOPMENT OF EXPERIMENTAL FATTY LIVER AND CIRRHOSIS*

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Introduction

Interest in the fatty livers which may be produced in experimental animals by feeding diets low in choline and its precursors (betaine, methionine, and methionine-containing protein) has extended in our laboratories at Toronto over a period which can be said to have originated with the discovery of insulin in 1922 and has continued up to the present time. The sequence of pathologic events which begins almost as soon as an animal is placed on the low-choline regimen, has now been studied in a large number of animals. Most of the early studies which resulted in the discovery by Best and his associates[2] of the lipotropic action of choline were carried out on dogs, but since the end of World War II, most of our experiments concerning the pathological changes in choline-deficient animals have been carried out on the rat. Other species of animals are similarly affected as will be reviewed by Professor Best and his co-workers in the next paper. Much of the available evidence indicates that man should probably be included in the list of animals susceptible to some of the pathologic effects of low-choline diets.

Some of the early morphological changes which affect the liver in dietary choline deficiency are those described by MacLean and Best in 1934.[15] In this paper were reported the appearances of livers of dogs fed diets low in choline and of those of control animals which had received the same basal diet supplemented with adequate amounts of the lipotropic agents. Photomicrographs were published in black-and-white, as well as colored drawings of some of the microsections. Detailed protocols of some of the animals were published in full. In this early paper are several features worthy of special mention here (FIGURES 1 and 2). The *intracellular* storage of fat within parenchymal liver cells is well illustrated and described. In addition, these photomicrographs show definite evidence of the *extracellular* phase of fat storage within what we now call fatty cysts. Furthermore, in two of the illustrations, there is evidence of early fibrosis in these livers, and in the protocols it is expressly stated (Dog V) that in some areas of the liver "the number of fibroblasts and leucocytes in these areas was abnormally large" and that "the liver cords, however, were separated by dilated capillaries and by areas of beginning fibrosis." These reports clearly foreshadowed the recognition four years later of cirrhosis in animals fed low-choline diets, by Chaikoff and Connor[3] in dogs, and by György and Goldblatt in rats.[6]

We have begun with this description, because many phases in the sequence of events which we are about to describe in the livers of choline-deficient rats were already evident in these earlier studies. In rats, similar phenomena

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* Much of the investigation reviewed in this paper was supported by the National Cancer Institute of Canada.

can be studied in a greater number of animals because the lesions make their appearance within shorter periods than is the case in dogs. All the findings to be set forth briefly below have been previously published[7, 8, 9, 11, 12] or are in press.[10] This paper will consist, therefore, of a summation of our conclusions based on these studies. Data concerning dietary methods, histological techniques and photomicrographic evidence are given in full in the publications mentioned.

1. Intracellular phase of the accumulation of abnormal fat in livers of choline deficient rats: "Intracellular lipohepatosis"

Within a few hours after allowing rats access to diets low in choline, stainable fat appears *within* many of the liver cells. The fat is clearly intracellular in position and, at this stage, is seen in greatest amount within parenchymal cells in centrolobular and nonportal regions. Stainable fat droplets may not accumulate in portal liver tissue until a later stage. Even such an early manifestation of the hepatic lesions of choline deficiency is in accord with the pathonomy that the effects of this dietary abnormality are always first observed and are present in greatest degree in the nonportal regions of the liver. This principle, as we shall see, holds true at all stages, including those of advanced cirrhosis.

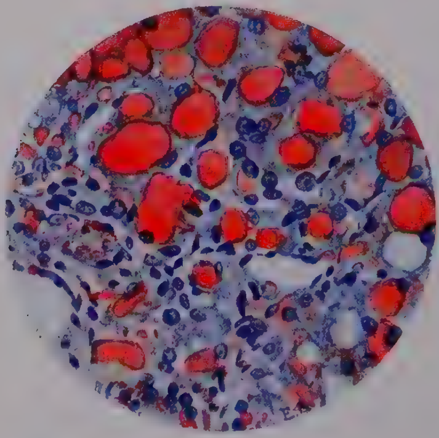

FIGURE 1. (Compare with FIGURE 2.) Early fibrosis in dogs resulting from atrophy and fibrotic replacement of fatty cysts. Figure 5B in MacLean and Best.[15]

Many terms have been used in the past to describe the abnormal accumulation of fat in the livers of choline-deficient animals. Such have included fatty change, fatty infiltration, and fatty degeneration. In our opinion, none of these phrases is free from ambiguity when used to describe the lesions in hepatic hypocholinopathy. Fatty change might imply merely a change in the state of the liver lipids, but we know[1] that the fatty droplets represent an accumulation of excess fat brought to the liver from the ingested food. The fat is initially held within liver cells and not in adipose tissue cells between them, as the term fatty infiltration would imply. The liver cells which contain the fat need not (and frequently do not) show any associated degenerative changes as is implied by the term fatty degeneration. If we wish to be precise in describing the state of the liver in early stages of choline deficiency, we can say that there is an accumulation of excessive amounts of stainable fat within hepatic parenchymal cells. For convenience, therefore, we suggest the term *lipohepatosis* for this condition. The phase described in this section is that of *intracellular lipohepatosis*. This is followed by *extracellular lipohepatosis* of which the cardinal feature is the formation of fatty cysts.

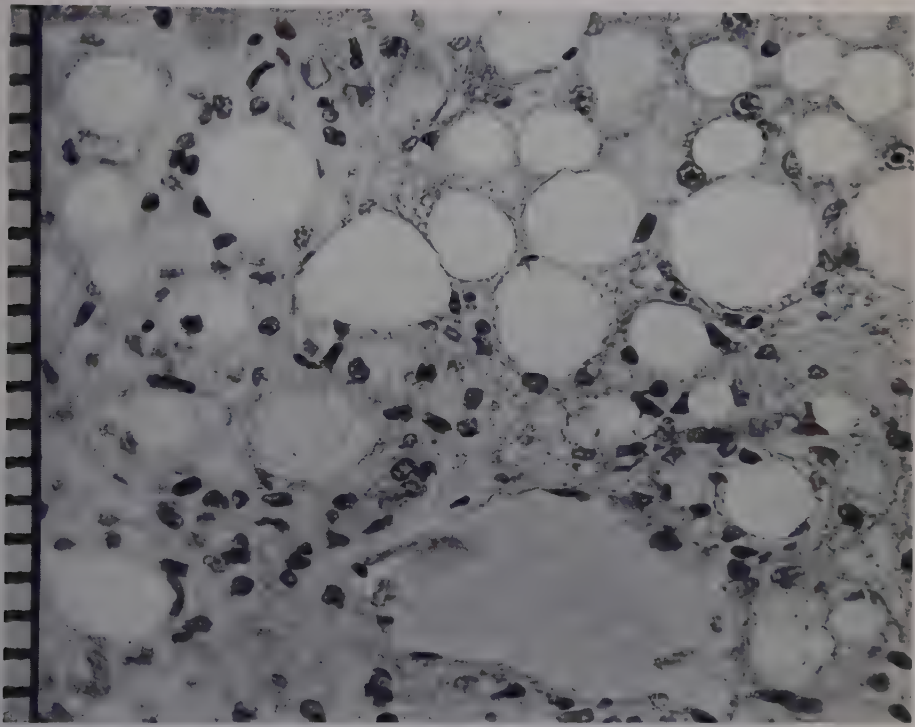

FIGURE 2. (Compare with FIGURE 1.) Region in choline-deficient rat's liver in stage of early fibrosis. Note fatty acids undergoing atrophy and fibrotic replacement. Paraffin section; hematoxylin and eosin stain. Divisions of scale at left are 10 micra apart.

2. *Extracellular lipohepatosis; formation of fatty cysts*

If the condition of dietary choline deficiency is allowed to continue unchecked in rats, fat accumulates within cells to such an extent that many are overloaded to the bursting point and rupture. Again, this occurs initially and to greatest degree in the nonportal regions. At first, the released fat which thus escapes from within cells is still surrounded by the latter and is therefore contained as relatively large pools of lipid within simple epithelial cysts. The cysts are formed in the following manner: Progressive enlargement of fat globules within neighboring liver cells, compresses the adjacent intervening segments of their limiting cell-membranes (which have been caught between the globules) into morphologically single, tenuous septa. By its formation, such a septum conjoins the parent cells but still separates the adjacent fat globules and prevents them from coalescing. If the globules continue to enlarge, they eventually so stretch the interposed septum that it is torn. As a result, the fat globules in the torn cells coalesce. The resultant pool of fat is enclosed, however, by the crescentic remnants of the cytoplasm of the parent group of cells which are still linked together. The nuclei of the cells appear essentially unaltered despite the fact that the limiting membranes of the cytoplasm have been torn. The pool of fat, that has thus been formed by coalescence of the lipid globules released from the torn cells is extracellular. It does not lie freely in the liver tissue but is contained within a simple, epithelial cyst. Fatty cysts, once formed, may continue to enlarge and, in turn, fuse in the same manner as did individual cells with other adjacent cysts or with single, fat-laden cells. Eventually, however, rupture of large cysts allows escape of fat from the liver by pathological routes to be described. When fat is no longer either intracellular or intracystic, it appears to constitute a grave menace to the health of the animal.

3. Extracellular lipohepatosis; rupture of fatty cysts and formation of trabeculae

It has been observed repeatedly that the distribution of fatty cysts in the livers of choline-deficient rats presages that of the fibrous trabeculae which are formed later. This juxtaposition is not merely fortuitous, for the evidence clearly indicates that it is the dissolution of ruptured cysts that produces the trabeculae. Fatty cysts form, initially, as already mentioned, around the centrolobular radicles of the hepatic vein. From here, they extend in annular chains which link adjacent central veins. They apparently follow this course by virtue of the fact that, if they extend into any other portion of the lobular parenchyma, they will be advancing more or less directly towards nearby portal areas. The progressive sites in which fibrosis develops are identical with those in which cysts previously have formed. Trabeculae result from condensation of the parenchymal and stromal remnants of ruptured fatty cysts. This condensation of cyst-remnants occurs whenever the cysts lose their fatty contents. Rupture of cysts over 100 micra in diameter (as measured in paraffin sections) is apparently nearly always accompanied by escape of fat. Large cysts are covered with a fine network formed of bile capillaries and sinusoids which originally ran between the liver cells that have taken part in the formation of the cyst-wall. The larger the cyst, the more extensive this network, and accordingly the more likely that some component of the latter will be torn with rupture of the cyst-wall. As a result, free communications may be established between the lumen of the cyst and those of sinusoids or bile ductules. Fat may be drained from the cyst by either of these two routes, and the remnants of the emptied structure condense into a fibrotic residuum. In this manner, the annular chains of cysts connecting adjacent centrolobular regions progress into the fibrous trabeculae familiar to anyone who has observed early stages of experimental dietary cirrhosis in animals.

4. The development of a "portal" distribution of the fibrous tissue

In their initial reports György and Goldblatt[6] interpreted the distribution of fibrosis in choline-deficient rats as portal. They were studying late stages of the condition. Although it is now clear from the work of Lillie *et al.*,[14] Glynn *et al.*,[5] and from the foregoing description that, in the early stages, the trabeculae are nonportal, later fibrosis clearly develops around large, easily identified, portal triads. In this sense, the cirrhosis is unquestionably portal in distribution. But the fibrosis in these sites replaces parenchyma which, although adjacent to *conducting* portal vessels (Elias), is not periportal in the sense that it is supplied by blood which has not previously come into intimate contact with liver cells. Parenchyma that lies beside these large conducting veins is supplied by sinusoids which do not frequently or necessarily communicate directly with the near-by veins. In most instances it has been shown[10] that these sinusoids receive their blood via others that in turn communicate with terminal *venules*, which actually perform the function of distributing the blood to the liver parenchyma. The large conducting veins are those which bring the blood to the terminal venules, and the latter empty the blood into sinusoids which rarely communicate directly with vessels of the order of size of veins. This is generally true of tissues and organs throughout the entire body; for example, in the kidney and pancreas, capillaries and sinusoids nearly always arise from venules rather than directly from veins. Portal venules lie in the center of a structural unit of liver tissue described by Rappaport and his co-workers as the *hepatic acinus*.[16] The periphery of an acinus may sometimes lie against a conducting vein and, hence, parenchyma in such a site, although periportal in a geographic sense, receives its blood via other sinusoids from a terminal venule. Such sites are, in terms of the entry of fresh blood into a hepatic unit, no more functionally periportal than are sites which are obviously closer to radicles of the hepatic vein. Fatty cysts and, subsequently, their trabecular remnants may therefore appear in these regions adjacent to conducting veins. Fibrosis in such positions at first appeared to constitute a paradoxical exception to the pathonomy that nonportal regions of the liver are those most severely affected in animals suffering from a dietary deficiency of choline. If the hepatic architecture is interpreted according to Rappaport's concept of the hepatic acinus, and in terms of the functional entry of fresh blood into sinusoids around terminal distributing venules, the paradox is resolved. It then becomes clear why initially nonportal lesions become geographically (only) periportal and surround large conducting portal triads. The latter structures are prominent even under the lower powers of the microscope and, unless the sites of the smaller, relatively inconspicuous terminal venules are examined with the aid of higher magnifications, it may not be realized that the adjacent parenchyma (which is *both* geographically and functionally periportal) is free from fibrosis. The term, portal cirrhosis, is acceptable at this stage from a purely descriptive standpoint, but it should be realized that the sites of fibrosis are actually nonportal with reference to blood supply. In microsections of cirrhotic livers obtained at autopsy from alcoholic patients, we have observed that the parenchyma around terminal portal venules is usually quite free of fibrotic lesions,[10] although the large conducting veins in these cases frequently were surrounded by pathological amounts of fibrous tissue. This suggests that the distribution of the trabeculae in alcoholic cirrhosis in man is also periportal only in a geographic sense and that, in this respect as well as perhaps in others, dietary cirrhosis in choline-deficient rats is the morphologic counterpart of that found in alcoholic man.

5. *Nodular hyperplasia*

After four or five months of choline deficiency, focal areas of hyperplasia throughout the liver become prominent features in microsections. In these areas which surround the terminal portal venules, mitotic figures may be found with ease. Frequently, cells with two, three, or even four nuclei may be encountered. As a result of this proliferation, nodules of compensatory parenchymal tissue are formed and they project, on the surface of the organ, thus producing the characteristic hobnail appearance which is such a striking feature of these livers when examined grossly. Growth of the nodules results in com-

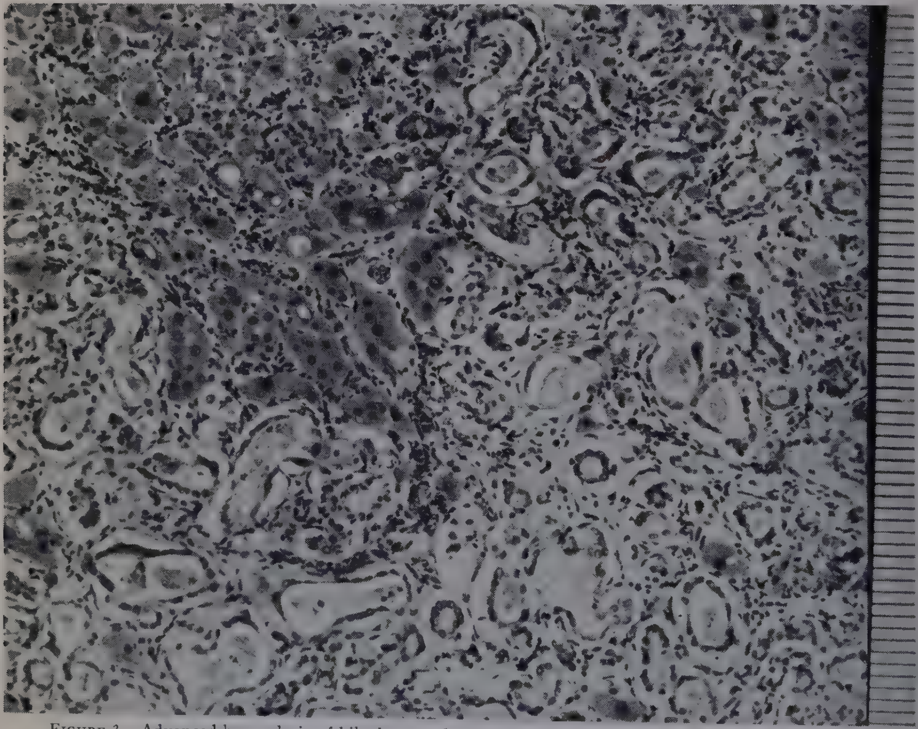

FIGURE 3. Advanced hyperplasia of bile ducts and regenerating liver cells in choline-deficient rat's liver in stage of late, nodular cirrhosis. This field may represent precancerous or early neoplastic change. Paraffin section; hematoxylin and eosin stain. Divisions of scale are 10 micra apart.

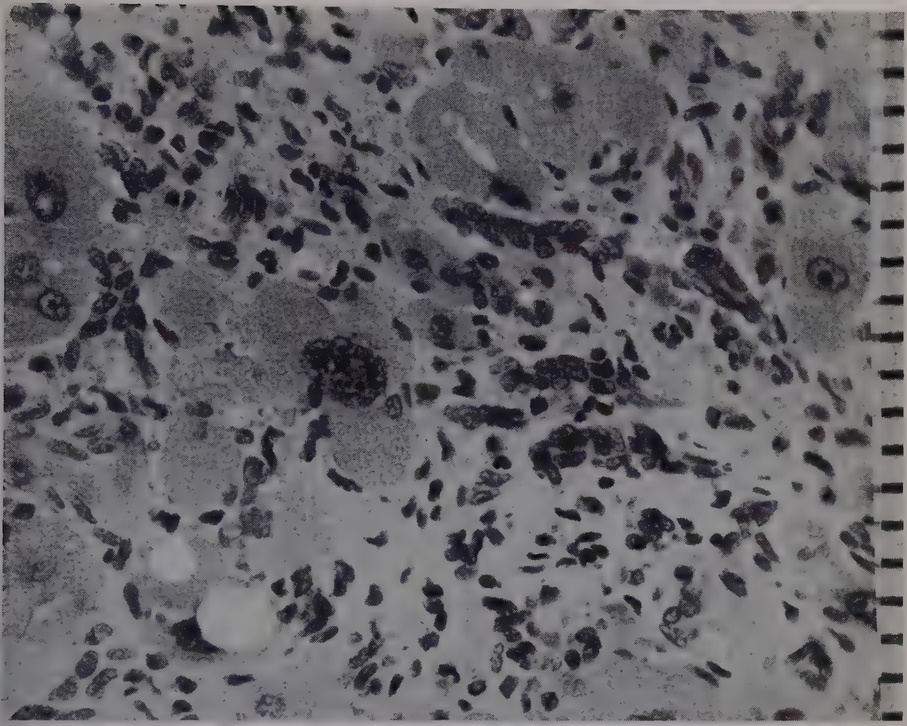

FIGURE 4. High-power view of small area from section of liver illustrated in FIGURE 3 at lower magnification Note the bizarre shape of the nuclei and the proliferation of bile duct epithelium. Divisions of scale are 10 micra apart.

pression of the surrounding fibrous trabeculae. Even though the animals are continued on the low-choline regimen, the newly formed tissue rarely becomes as fatty as that previously present. In the regions of compensatory proliferation, the terminal vessels may lengthen and divide. Studies of the arterial supply of the large nodules have not yet been made.

6. *Neoplasia*

At the edges of the nodules, as well as within their substances in some instances, isolated liver cells may assume bizarre forms with rather wide variations exhibited in their size, shape, and staining reactions (FIGURES 3 and 4). In our experiments, this appearance rarely suggests anything more than very active hyperplasia. There is, however, little doubt that in susceptible strains of rats, cirrhosis induced by low-choline diets may progress to true neoplasia. The evidence for hepatic tumor formation in choline-deficient rats is presented in a paper by Doctors Copeland and Salmon who first reported its occurrence.[4]

Conclusions

The escape of fat from cysts into the vascular system results in intermittent or chronic episodes of fat embolism. These emboli are carried to the heart, lungs, and kidneys where microscopic evidence of their damaging effect has been noted.[8, 9] Other organs may well be involved in this phenomenon, and it is possible that the arteriosclerotic changes in the aorta, carotid arteries, and coronary vessels which we have reported in choline-deficient rats[12] may be another result of fat circulating in the blood stream in this obviously pathologic state.

We have followed the regressive changes which regularly develop in fatty hepatic cysts of previously choline-deficient rats when lipotropic factors are restored to the animals' diets.[13] Intracellular fat is mobilized within a matter of hours, thus rendering the cysts particularly prominent. Weeks, and, in some instances, months, of choline administration may be required before the fat within cysts is absorbed by the cells forming their walls. Once lipid regains this intracellular position, dietary choline rapidly effects its disappearance. The fact that cysts may remain for relatively long periods in previously fatty livers even after therapeutic measures have removed all intracellular, stainable fat offers a diagnostic aid in the identification of fibrosis of those types which may have been initiated by lipohepatosis. We have examined sections from one hundred consecutive, unselected cases of cirrhosis coming to autopsy in the Department of Pathology at the University of Toronto, in an effort to evaluate the possible diagnostic significance of residual cysts within fibrous trabeculae. Almost without exception, these intratrabecular cysts were present in cases of cirrhosis in which the history clearly indicated that they had been associated at some time with lipohepatosis.[9]

Fatty cysts are the cytometaplastic links between lipohepatosis and cirrhosis. In miniature, they reflect the gross structural changes and biochemical findings in livers of choline-deficient rats at all stages. As the livers become increasingly fatty, fibrosis makes its appearance. Our evidence indicates that formation and rupture of fatty cysts is, by condensation of their remnants, the direct cause of fibrosis. As the livers become increasingly fibrotic, their total fat content, as assayed biochemically, decreases. This phenomenon is explained by the concept that the formation of the fibrous trabeculae is dependent on the escape of fat from the cysts and thus eventually from the liver via either the biliary or vascular systems. These events would lead one to expect the total fat content of the liver to vary inversely with the amount of fibrous tissue, as is the case. This relationship is probably emphasized by the fact that new tissue in regeneration nodules rarely undergoes appreciable fatty change. The concept of the formation and natural history of fatty cysts permits an appreciation of the pathogenic relation between excessive fat and fibrosis in livers. Formation of fatty cysts and condensation of their ruptured remnants into fibrous trabeculae implies, *ipso facto*, that the fibrosis is the direct result of overloading focal regions of the liver parenchyma with abnormal fat. It is apparent from this that the mere presence of even appreciable amounts of fat in the liver will not necessarily lead to fibrosis. Fibrosis will result only if the accumulation of fat is so great that rupture of cells produces cysts which, in turn, disintegrate. This explanation may serve to help resolve some of the apparently conflicting conclusions which have been previously drawn concerning the cirrhogenic nature of lipohepatosis.

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Discussion of the Paper

Doctor I. N. Dubin (*Armed Forces Institute of Pathology, Washington, D. C.*): In the Registry of Hepatic Pathology we have had the opportunity of seeing a large number of cases of human liver disease studied by needle biopsy and, on the basis of this experience, we have been led to conclusions which are somewhat at variance with those of Doctor Hartroft. We have now seen biopsy material from over 400 cases of portal cirrhosis (*i.e.*, so-called nutritional or alcoholic cirrhosis) and from a similar number of cases of fatty metamorphosis with or without fibrosis. We should summarize our experience as follows:

(1) With rare exceptions, we have not observed "fatty cysts" in man comparable to those described in rats by Doctor Hartroft. When present, the cysts were minute, extremely few in number, and not related to fibrosis.

(2) On the contrary, we are of the opinion that fatty metamorphosis of itself does not lead to hepatic necrosis or cirrhosis. We have seen cases of fatty

liver studied by serial biopsies which at the end of two or three years still failed to show significant portal fibrosis, let alone cirrhosis.

(3) In our experience, fatty metamorphosis of the liver in any one patient may come and go without appreciably affecting the degree of portal fibrosis or cirrhosis.

(4) We believe that the prime lesion in the pathogenesis of human portal cirrhosis (alcoholic cirrhosis) is *necrosis*, not fatty metamorphosis. The presence of fat is merely a parallel phenomenon. The same factor or combination of factors which causes the necrosis also happens to cause fatty metamorphosis. Fatty metamorphosis without the basic factor of liver necrosis does not lead to fibrosis. On the other hand, we have seen extensive necrosis of liver without appreciable fatty metamorphosis; yet this will be followed by cirrhosis.

This type of cirrhosis comes about as the result of two main processes: increase of collagen and nodular regeneration of liver cells. (a) The diffuse necrosis of liver leads to a fairly diffuse but somewhat irregularly distributed increase in collagen, not only in portal areas but particularly in the parenchyma which it traverses in thin bands. (b) In the absence of the original reticular framework to guide the regenerating liver cells, the newly-formed parenchyma grows in the form of roughly spherical nodules without proper relationship to pre-existing key structures such as portal areas and central veins. Indeed, the intralobular bands of collagen may be partly responsible for this abnormal architecture by forming a new but haphazard framework along which the regenerating tissue grows.

(5) The acute necrotizing phase is characterized by a fairly diffuse degeneration and necrosis of liver cells, many of which are transformed into Mallory bodies. Accompanying this is a moderate amount of inflammatory reaction, scattered throughout the lobules and involving portal areas as well. Fatty metamorphosis may or may not be present. At this point, three things may happen: (a) If the liver damage is sufficiently severe, the patient may die in the acute phase from liver deficiency. (b) If the patient survives and mends his ways before he has subjected himself to too many bouts of liver damage, he will be left with only mild residual portal scarring. (c) If the hepatic necrosis is continual or recurrent, a well-defined portal cirrhosis will follow.

Doctor Hans Popper (*The Hektoen Institute for Medical Research, Chicago, Ill.*): I should like to assume a position somewhat between those taken by Doctors Hartroft and Dubin. Studies with Doctor Hans Elias based on three-dimensional reconstruction and geometrical analysis have convinced me that there are several pathways along which fatty liver may proceed to cirrhosis formation. One pathway is that which Doctor Hartroft has explored by his thorough and ingenious investigations. I feel, however, that this occurs mainly in the presence of very large fatty livers in which fatty cysts are found in large numbers and in which they may be even responsible for the marked hepatomegaly. Doctor Elias and I are not sure whether the fibrosis results from collapse of the fatty cysts or from the formation of collagen fibers in the vicinity of the cysts. The second pathway is the result of necrotizing and inflammatory changes complicating the fatty liver, somewhat similar to the processes which Doctor Dubin just described so well. The inflammation or the necrosis with associated inflammation leads to an irregular fibrosis with the formation of collagen fibers in the parenchyma. Moreover, radiating or stellate fibrosis of the portal triads develops. The latter may possibly be due to irritation by breakdown products drained from the parenchyma to the portal triads. In our opinion, however, the most important pathway results from stress within the parenchyma exerted from the vicinity of regeneratory, ordinarily fat-free, liver cell plates, usually several cells thick, and the fat-containing one-cell-thick plates. This stress produces planes of breakage in which fibers develop to form a straight septum, subdividing the lobule. In human fatty livers, where periods of nutritional disturbance alternate with at least partial recovery, these stress planes probably develop more readily. In conclusion, I believe that the degree of participation of the different pathways varies in the individual cases. This accounts for the polymorphic picture seen in the development of cirrhosis from the fatty liver in man.

Doctor Charles S. Davidson (*Thorndike Memorial Laboratory, Boston City Hospital, Boston, Mass.*): In studying alcoholics with acute liver disease at the Boston City Hospital, we have observed ubiquitous fatty changes in the livers, but we are not convinced that the fatty changes always constitute the most important pathological abnormality. A typical hepatic lesion characterized by parenchymal disorganization, intracellular hyalin, and necrosis frequently occurs and is distinct from the fatty lesion. This lesion correlates better than does fat with the acute severe liver impairment which often leads to fatal coma. The lesion seems to have a predilection for females. A summary of this work done in collaboration with Doctor Gerald G. Phillips, Dante Campagna-Pinto, and Frederick Parker, Jr., was published in the Proceedings of the American Federation for Clinical Research meeting for May, 1953.

Doctor Hartroft: In reply to Doctor Dubin's comment, I should like to emphasize the following points. We have examined nearly one hundred cases of cirrhosis in human patients and, in a very high percentage of cases associated with alcoholism, diabetes, obesity, and anemia, fatty cysts in various stages were found *within* fibrous trabeculae. Accordingly we have named cysts in this position *intratrabecular fatty cysts* and, from their position, have concluded that, as in the rat, these cysts may be cirrhogenic in nature. These results have already been published (Hartroft. 1953. Arch. Path. **55:** 63–69).

Doctor Dubin is of the opinion that fatty metamorphosis of itself does not lead to hepatic necrosis or cirrhosis and cites instances of fatty livers which he has followed by serial biopsies and which, at the end of two or three years, failed to show fibrosis or cirrhosis. We have never maintained that an abnormal accumulation of fat leads to necrosis, nor have we ever maintained that the mere presence of stainable fat in the liver leads to fibrosis. All our studies, both experimental and clinical, of the pathogenesis of dietary cirrhosis, lead us to believe that, not only must fat be present in the liver to produce fibrosis, but also it must be accumulating so rapidly and to such a degree that it ruptures cells, thus leading to parenchymal damage. The complete history of fatty cysts, their formation, rupture, atrophy, and condensation of stromal remnants into fibrous bands, describes a series of steps by which excessive accumulation of hepatic fat leads to traumatic rupture of distended cells and to fibrosis. We have seen many instances in rats where the conditions were such that the lipotropic deficiency, although severe enough to cause accumulation of fat in liver cells, was not sufficiently extreme to produce fatty cysts and their sequelae, including fibrosis and cirrhosis. It is equally true that, once cirrhosis is established, similar degrees of "nontraumatic" accumulation of fat could come and go, as Doctor Dubin states, without appreciably affecting the degree of cirrhosis.

In the experimental animal subjected to dietary choline deficiency (uncomplicated by a concomitant deficiency of tocopherol, cystine, and methionine), there is no doubt whatsoever that a cirrhosis results. In these animals, the consensus of opinion of those experimental pathologists who have studied the livers in detail is that necrosis of parenchymal cells is hardly ever seen. We are using the term necrosis here in the sense meaning acute or sudden death of tissue. It is apparent, however, that the slow death of cells or atrophy, associated with rupture of fatty cysts is, in a sense, necrosis, if the term is used with this broad meaning. We prefer, however, for the sake of clarity, to reserve the term necrosis for the sudden acute type of cell-death characterized by pyknosis, karyolysis, and karyorrhexis. In clinical cirrhosis, there is a possibility that alcoholism may be associated with deficiencies other than that of choline, but we have been able to duplicate the lesions of chronic alcoholics in livers of rats fed alcohol in the presence of no other known deficiency than that of choline and its dietary precursors (Best, Hartroft, Lucas, and Ridout. 1949. Brit. Med. J. **2:** 1001–1006).

Doctor Popper has excellently summarized the points regarding the pathogenesis of cirrhosis in man, and we find ourselves in agreement with many of his findings. We are indebted to Doctor Elias for his beautiful tri-dimensional reconstructions of fatty liver cysts in alcoholic man.

In all attempts to correlate the amount of fatty change with the amount of fibrosis and/or cirrhosis in sections, whether of the experimental animal or alcoholic man, it is well to emphasize that there is abundant evidence that the greater the fibrosis the less the amount of fat. This inverse correlation is again reflected by the "life story" of fatty cysts seen in livers of choline-deficient rats. The more cysts which rupture, thus allowing their contained fat to escape from the liver by one or more of the pathological routes we have described (Hartroft, W. S. & J. H. Ridout. 1951. Am. J. Path. **27:** 951–991), the greater the fibrosis resulting from their condensed parenchymal and stromal remnants and the less the amount of fat which will remain in the liver. New liver tissue in the regeneration nodules never becomes very fatty, so that the total fat content diminishes with rupture of cysts and advance of fibrosis and cirrhosis. If, in addition, the animal becomes sick and fails to eat for a period before death, or if it is treated with adequate diet and lipotropic factors, much of the fat in the cirrhotic liver will be mobilized (Hartroft & Sellers. 1952. Am. J. Path. **28:** 387–399). Residual intratrabecular cysts may not be numerous and almost certainly will be small, but in the experimental animal where every stage of the fat accumulation and subsequent cirrhosis has been followed, it is clear that these few persistent cysts represent the stigmata of a liver that was previously fatty to the "bursting point." Similar findings in man may have the same significance and may, therefore, provide important diagnostic evidence suggesting the etiology and pathogenesis of the cirrhosis manifest at autopsy. We believe that, if these points are kept in mind, much of the apparent confusion concerning the pathogenic relationship of fatty to cirrhotic livers might be resolved, not only in experimental animals, but also in man, including perhaps some of Doctor Davidson's cases.

THE LIPOTROPIC FACTORS

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The nutritional role of choline was discovered[1,2] in 1932, so that this year it comes of age as a dietary factor. Choline was the first of the accessory food factors which was shown to protect the liver; *i.e.*, in its absence from the diet, pathological changes appear promptly in the hepatic cells. The work on choline, as a survey of the literature clearly shows, stimulated great interest in liver fat, in the effect of large accumulations of fat on hepatic function, and in the part played by other dietary factors in the protection of the liver.

The very general and broad title assigned to us here has made us somewhat uncertain about what is desired. The topics to be dealt with by our learned colleagues on this comprehensive program leave us but little in the way of definite responsibility, and we are therefore quite free to discuss certain general aspects of our subject.

We are all familiar with the fact that choline, betaine, methionine and, under some conditions, vitamin B_{12} and inositol, protect rats in varying degrees against the development of fatty livers. In the absence of choline from the diet, many species of animals have been found to develop this abnormality. The rat,[1,2] dog,[3,4,5,6] mouse,[3,7] rabbit,[8] hamster,[9] calf,[10] pig,[11] and duckling[12] exhibit fatty livers when the diet lacks sufficient of the lipotropic agents, choline or its precursors. Some of the classical lesions are illustrated in Professor Hartroft's presentation (page 633). In some species, such as the guinea pig, it is very difficult to produce an abnormal deposition of fat in the liver by feeding diets low in choline.[13] We must always keep in mind that interference with the function of hepatic cells by means other than deficiency of the lipotropes may lead to fatty livers. Fatty livers of guinea pigs on scorbutic diets[14] are probably not attributable to a lack of dietary choline.

Many readers are doubtless familiar with the hemorrhagic kidney syndrome[15] which may be consistently produced in young rats on diets low in the lipotropes. With the hypolipotropic diets currently used in our laboratory we are able consistently to produce kidney lesions in *mature* animals. Somewhat similar renal lesions have been reported in young pigs[16] and calves.[10] The failure to produce this kidney lesion in young mice and puppies, both of which develop fatty livers readily enough, reveals a problem which may possibly require extended study. A comprehensive investigation and comparison of the relevant enzyme systems will undoubtedly help. An interesting sequel to the renal lesion in rats is the appearance in later life of hypertension.[17] This has not yet been reported in other species. You are also familiar with the failure of baby chicks,[18] turkey poults[19] and ducklings[20,21] to grow and their tendency to develop slipped tendon disease on choline-deficient diets. In our early studies on the effects of choline in preventing and curing the pathological changes in the diabetic dog, we mentioned "areas of beginning fibrosis" which did not appear to be as favorably affected by choline as the fatty changes,[22] but we did not observe cirrhosis until after the work of Chaikoff and Connor[23] on dogs and György and Goldblatt[24] on rats. The role of choline deficiency in the production of fatty livers and cirrhosis in man is less clear cut. The crucial experiment to determine the lipotropic activity of choline in man has not yet been performed. The nearest approach is that of Phillips, Gabuzda, and Davidson.[25]

By these brief references to the effects of choline and its precursors in various species, we have been leading up to reconsideration of the word "lipotropic." What should be included under this term? The word was introduced to describe substances which decrease the rate of deposition and accelerate the rate of removal of excess liver fat and was so defined by Best, Huntsman, and Ridout.[26] While the word *dietary* was not specifically mentioned, it was obvious from the context that the active principle under discussion was a component of the diet. The word lipotropic has been carelessly used, but some of the misuse is perhaps due to real difficulties in deciding on the proper scope of the term. There is, of course, no justification whatever for many of the misapplications; *e.g.*, a change in lipid content of a fat-laden pterygium on the cornea or in the amount of depot fat in obese human subjects or in animals. The term lipotropic is not applicable to changes in lipid concentration in the blood or generally in the body. The word does not apply to *increases* in liver fat, which at least one writer has suggested. The original definition stated that the term refers to *decreases* in fat deposition and is applied to changes in the *liver*. At one time, however, we[27] suggested an extension of this to include the other established effects of choline and its precursors, *viz.*, prevention of renal lesions and of cirrhosis. Whether this proposal was wise has since been debated many times in our laboratory. Some of these changes may include effects involving fat deposition; *e.g.*, in the kidney[28] or perhaps in the walls of blood vessels. If it is firmly established that the primary, or a highly significant, part of the process initiated by choline in organs other than the liver is essentially similar to that in the hepatic cells; *i.e.*, involving the prevention of excess fattiness. The term lipotropic may then be extended to include these other lesions.

Let us compare the antiperotic action of choline and its effect in preventing the renal hemorrhagic syndrome. We can find no justification for calling the antiperotic effect lipotropic; but if prevention of deposition of excess fat in the proximal renal tubules is the essential process, this protection of the kidney may be considered a lipotropic phenomenon.

On the other hand, although the growth effect of choline, which can be clearly demonstrated under certain conditions, is mediated through biochemical processes intimately interrelated with lipotropic action, this action should be designated by a term other than lipotropic.

We should stress here that a substance may exert a lipotropic effect without being a lipotropic agent. It might increase the rate of formation of, or potentiate in some way the action of, a lipotropic agent.

The role of antibiotics, such as aureomycin, in lipotropic phenomena is not too clear. Doctor György will discuss these in detail. We feel that they have not qualified as lipotropic agents although they do modify the requirement for choline and methionine. Thiamine, calcium, vitamin A, or any other dietary essential, in whose absence the food intake and rate of growth are affected, may also affect the choline requirement. We cannot call all the essential food factors antilipotropic agents. The lipotropic effect of the antibiotics cannot be due to change in food intake, as György has shown, but there is still a possibility that a change in absorption may play a role. The consideration of the antibiotics later in this monograph is of pertinent interest.

To continue our discussion of the term lipotropic, since a fatty liver is always a forerunner of the cirrhosis of choline deficiency, one can properly call the *preventive* effect of choline a lipotropic effect. Can one say, however, that the curative action of choline on a fatty cirrhotic liver is partly or wholly lipotropic? It would appear certain that a part of the reticulum or fibrotic tissue actually disappears as an end result of choline action, but it can not be stated at present whether this result is secondary to an effect on fat, to the regeneration of hepatic cells which the disappearance of fat may make possible, or to a direct attack on the fibrous tissue.

Another interesting question is raised by the finding[29, 30] that, under certain conditions, large accumulations of fat may persist for long periods in the liver without the appearance of fibrosis. In our own experience, this has been observed in experiments in which vitamin B_{12} prevented the development of cirrhosis. This effect of vitamin B_{12} was first reported from György's laboratory, but the details of this particular aspect of their studies have not yet been published. Doctor Drill will be discussing the role of vitamin B_{12} in lipotropic phenomena. We should like to mention, however, our recent finding that rats may be maintained with vitamin B_{12} on low choline diets (9 per cent protein and very low in methionine) for 16 months without cirrhosis but with fairly large amounts of fat in their livers (12 to 15 per cent). In 22 animals not receiving B_{12}, all developed cirrhosis between the 3rd and 6th month and their livers were more fatty (15 to 30 per cent) than those given B_{12}. The 87 animals receiving B_{12}, with one exception, were not cirrhotic. This is in confirmation of György's[31] original report. This raises the question: does vitamin B_{12} specifically prevent the cirrhosis, or does it act by increasing the biosynthesis of choline? It would be interesting to determine if a small supplement of choline which maintained the liver lipids at levels comparable to those secured with B_{12} would have the same anticirrhotic effect. If this proved not to be so, could it be that vitamin B_{12} acts by increasing the rate of turnover rather than merely reducing the amount of liver fat?

In confirmation of Salmon, Copeland, and their colleagues,[32, 33, 34] we have seen a dramatic effect of vitamin B_{12} on the renal lesions in young rats, yet in others on similar diets with a lower "lipotropic background," no beneficial effect whatever from B_{12} was observed. Similarly, in older rats, others have demonstrated that, by alteration in the dietary background, one may either reveal or fail to elicit the lipotropic effect of vitamin B_{12}.[31, 35, 36, 37, 38]

In attempting to account for these observations, we have all been forced to recognize the importance of several nutritional dilemmas. The first of these has been appreciated for some time and will be discussed more fully later;

namely, that the ideal basal hypolipotropic diet should contain no methionine. The second dilemma is of a similar nature but more involved, since it concerns the relationship of vitamin B_{12} to methionine and choline.

It became apparent quite early that the nature and amount of protein in the diet of a rat or dog affects the choline requirement, but to this day no adequate systematic study of proteins for use in designing hypolipotropic diets has been published. In several laboratories, including our own, a few purified proteins and a few crude proteins have been fed in attempts to find an ideal diet for studies of lipotropic phenomena and of other phenomena associated with choline deficiency. For many years now we have felt that the diet should be complete in every respect, except for substances possessing lipotropic activity. In spite of the ubiquitous nature of choline, the preparation of diets essentially free from choline, betaine, inositol, and vitamin B_{12} offers no serious problem; but to supply the essential amino acids without choline-precursors is a problem of the highest order. While it is true that no proteins free from methionine are known that are readily available in quantity, the major difficulty is the nutritional dilemma rather than the question of supply. Because methionine contains a labile methyl group which may be used for the biosynthesis of choline, one would like to eliminate methionine from the basal diet. It is well known, however, that complete removal of any essential amino acid from a diet leads to loss of appetite. It is known also that, when the caloric intake is low, even severely hypolipotropic diets do not produce fatty livers. Thus a diet *low* in methionine seems more suitable for lipotropic studies than one completely free from it.

Two chemical procedures are available for eliminating methionine from proteins. Welch[39] hydrolyzed casein with hydriodic acid to demethylate the methionine. After removal of most of the hydriodic acid by evaporation *in vacuo*, and of the rest with silver carbonate, the remaining amino acids were obtained for feeding by evaporation of the solution. The second chemical method of obtaining a diet free from methionine, devised by Toennies,[40] depends upon the fact that hydrogen peroxide converts methionine, even in the intact protein, to the sulphone which is inert nutritionally.

To prepare diets low in methionine one may utilize the proteins of peanuts, peas, or soybeans. The proteins may be isolated from these seeds and fed with other pure chemical substances in synthetic diets; or the meals themselves may be extracted with warm 70 per cent alcohol to remove choline and betaine and, combined with minerals, vitamins, fats, and carbohydrates to produce hypolipotropic diets. TABLES 1 and 2 show a few of the protein mixtures which we have used in our laboratory to produce hypolipotropic diets causing fatty livers, hemorrhagic kidneys, or cirrhosis.

To each of these protein mixtures, the same amounts of minerals, vitamins, fats, and carbohydrates were added to produce choline-free diets with essentially the same total protein content and the same total methionine content. Yet Diet 2 proved, as we anticipated, to be more severely hypolipotropic than did Diet 1. The first diet, although it contains 20 per cent of protein, is inadequate with respect to tryptophane, threonine, and phenylalanine. Rats consuming Diet 1 barely grow at all. The much better assortment of essential

TABLE 1

Diet 1		*Diet 2*	
Casein	8%	Casein	6%
Gelatin	12	Peanut meal (Alc. ext'd.)	30
Total protein	20	Total protein	21
Total methionine	360 mg.	Total methionine	360 mg.
		(Copeland & Salmon[41])	

amino acids in Diet 2 permits good growth. But when growth is improved, the choline requirement is increased and, at the same time, the amount of dietary methionine available for lipotropic purposes is diminished. Thus the second diet produces fatty livers of greater severity than does Diet 1 and produces them more rapidly. The increased rate of production and greater severity of the lesions caused by Diet 2 is seen most dramatically in the development of hemorrhagic kidney lesions in weanling rats.

TABLE 2 presents the composition of the protein moiety of several other diets that have been used in our laboratory when still more severely hypolipotropic conditions are desired.

The last diet shown in TABLE 2 contains less than one third the quantity of methionine present in the widely used and severely hypolipotropic mixture of 30 per cent peanut meal (extracted with alcohol) and 6 per cent casein suggested by Copeland and Salmon.[41] Diet 6 causes very severe signs of choline deficiency. We wish to emphasize once more that diminishing the methionine content of a diet by reduction of the protein will not necessarily intensify the hypolipotropic effect. A ration containing 4 per cent of casein, which has been used by many workers, is not nearly as hypolipotropic as one containing 20 per cent arachin or as one containing 12 per cent arachin with 2 per cent fibrin or 8 per cent peanut meal plus 8 per cent of soybean protein. Yet all contain the same amount of methionine (about 120 mg. per 100 gm.). Dietary adequacy with respect to other essential nutrients affects profoundly the requirement for lipotropic factors, an obvious fact the importance of which we and many others were slow to appreciate.

When one attempts to compare the curative properties of lipotropic agents and proteins in experimental or clinical cirrhosis, one really needs at least four groups of experimental animals. The characteristic features of the four dietary regimens to be compared are shown in TABLE 3.

A protein mixture for the first diet (A) might be 6 per cent alcohol-extracted peanut meal plus 6 per cent soybean protein. This contains 9 per cent of total protein with only 96 mgm. of methionine per 100 gm. of ration. Addition of 0.4 per cent of DL-methionine and 0.35 per cent choline chloride would give a diet (B) in which the total protein is low but the supply of lipotropic agents is adequate. A high protein diet, low in lipotropic factors (C) could be based on 50 per cent of alcohol-extracted peanut meal plus 5 per cent soybean protein (total protein 30 per cent, methionine 350 mg.). To produce Diet D one might add 150 mgm. DL-methionine (making the total methionine about 500 mgm.,

TABLE 2

Diet 3		*Diet 5*	
Arachin	12%	Arachin	12%
Gelatin	6	Fibrin	2
Casein	3	Casein	1
Fibrin	1		—
	—		
Total protein	22 %	Total protein	15%
Total methionine	250 mg.	Total methionine	150 mg.
Diet 4		*Diet 6*	
Peanut meal (Alc. ext'd.)	12%	Peanut meal (Alc. ext'd.)	6%
Soybean protein	12	Soybean protein	6
	—		—
Total protein	18 %	Total protein	9%
Total methionine	192 mg.	Total methionine	96 mg.

TABLE 3

A Low protein Hypolipotropic	*C* High protein Hypolipotropic
B Low protein Lipotropic	*D* High protein Lipotropic

as in Diet B) and 0.35 per cent choline chloride. Such a diet would ensure lipotropic adequacy. Omission of any one of the groups makes a valid comparison of the effect of protein *per se* with that of lipotropic agents, in the treatment of cirrhosis, impossible. No study of this type has yet been published.

Ideally, four more groups should be included, as shown in TABLE 4. These would permit us to separate out even more clearly the therapeutic factors of greatest influence. By low quality proteins, we mean in this connection those poor in methionine. A number of experiments along such lines were performed in our laboratory several years ago. Others are currently in progress and more are planned. In practically all of these we have tried to use diets in which all the essential amino acids were present in amounts sufficient to permit maintenance and repair of the tissues. We now look askance at curative experiments conducted with diets extremely low in protein since these obviously could not provide the building blocks needed for repair. Addition of protein to such a basal diet supplies the building blocks which choline alone cannot. Obviously, when good protein, which contains methionine, is fed, both the building blocks and a source of choline are supplied simultaneously. From the therapeutic point of view, this is ideal, but scientifically it is of no value in elucidating the fundamental mechanisms involved. Protein building blocks without choline or its precursors have always failed to give protection and, in cirrhosis caused by carbon tetrachloride, were shown to possess no curative value.[27]

TABLE 4

E	*G*
High protein (low quality) Extra methionine (DL)* No added Choline	Better quality protein No *free* methionine added No choline added
F	*H*
High protein (low quality) No extra methionine Choline chloride	Better quality protein Choline chloride

* An amount necessary to make the total methionine in Diets E and G equal.

We attempt to resolve the first dilemma mentioned earlier by providing barely enough methionine to meet the requirements for growth and make the unsupported and, we believe, unwarranted assumption that none would be used for synthesis of choline. This dilemma is further complicated by the well-established increased need for lipotropes when the growth rate is augmented by adding methionine. We should attempt to meet the more complicated second dilemma by using just enough methionine to eliminate the first dilemma and just sufficient vitamin B_{12} to satisfy other than lipotropic requirements; *i.e.*, trusting that none of either the vitamin B_{12} or methionine is "misappropriated" for lipotropic purposes. The experimental evidence suggests that this ideal also may be impossible of attainment.

One practical approach may be to conduct this type of experiment in mature and in growing animals and to compare the results. If one could find a species in which the transfer of methyl groups from methionine to choline did not occur, but in which all the signs of choline deficiency developed, our first dilemma, and part of the second, would be avoided. Failing this, we must try to find an otherwise innocuous chemical that blocks the enzymes transferring the methyl group of methionine to form choline. Even more helpful would be the discovery of a substance that would selectively block methylation of aminoethyl alcohol. This would resolve our present dilemmas and permit a breathing space until some new complications are revealed.

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LIPOTROPIC EFFECTS OF VITAMIN B_{12} AND OTHER FACTORS

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Our interest in lipotropic substances other than methionine, casein, or choline followed the publication of studies of Gillman and Gillman on fatty livers in children with pellagra.[1] By means of serial biopsies of the liver, they were able to demonstrate a marked lipotropic of hog stomach (Ventriculin). They also reported a partial lipotropic effect of liver extract and a minimal effect of brewer's yeast.

A survey of the literature showed a lack of knowledge concerning the lipotropic effect of such supplements in experimentally induced liver injury. Hog stomach had not received any experimental study. With regard to liver extract, an early study by Rhoads and Miller[2] reported that liver extract only partially restored to normal the abnormal liver function produced in dogs by a black-tongue diet. In 1942, György and Goldblatt[3] studied the effectiveness of a liver concentrate in preventing dietary cirrhosis in rats and concluded that the "liver extract was completely ineffective in preventing hepatic injury." The liver concentrate used in this later study was not commercial liver extract but the fraction of an aqueous liver extract soluble in alcohol. Yeast had received somewhat greater study but the results were conflicting.[3, 4, 5] Our first study, therefore, concerned the possible lipotropic effect of hog stomach, liver extract, and yeast.

The fatty livers were induced by a normal-protein—high-fat diet or a low-protein—low-fat diet (TABLE 1). Each rat also received a daily oral supply of the B vitamins as follows:

thiamin	20 μg.
riboflavin	25 μg.
pyridoxine	20 μg.
calcium pantothenate	100 μg.

Vitamins A and D were supplied by adding 5 drops of oleum percomorphum per kilo of diet.

In the first study, the following supplements were administered to rats receiving the high-fat or the low-protein diet: (a) crude liver extract (Lilly, 1 U.S.P. unit per cc.), 1 cc. subcutaneously 3 times a week; (b) brewer's yeast, 0.25 gm. per rat per day, mixed with the diet; (c) hog stomach (Ventriculin), 0.5 gm. per rat per day, mixed with the diet. Control groups of rats received the following: (a) choline chloride, 40 mgm. per day subcutaneously; (b) DL-methionine, 40 mgm. per day subcutaneously. One group receiving the high-fat diet received 8 per cent additional casein in the diet, the content of corn starch being decreased by 8 per cent. Similarly, with the low-protein diet, one group received an additional 0.5 per cent casein in the diet to control the small amount of protein added by the various supplements.

Untreated rats developed a marked fatty change, followed, depending on the length of the study, by fibrosis. The complete effectiveness of the choline

TABLE 1
EXPERIMENTAL DIETS

	Control diet, %	Diet 1, %	Diet 2, %
Casein	16	16	6
Lard	6	51	6
Cornstarch	60	30	70
Sucrose	15	—	15
Salt mixture	3	3	3
	100	100	100

TABLE 2
EFFECT OF VARIOUS SUPPLEMENTS ON FATTY LIVER

Group	No. animals	Liver fat gm. %
Exp. 1, 200 days (diet 16% protein, 51% fat)		
High fat	13	20.5 ± 1.72
Ventriculin	10	21.7 ± 1.38
Methionine	10	17.8 ± 2.40
Casein, 24%	9	15.4 ± 1.11
Yeast	10	11.2 ± 0.92
Liver extract	10	8.8 ± 0.65
Choline	10	7.3 ± 0.64
Exp. 2, 200 days (diet 6% protein, 6% fat)		
High fat	4	23.1 ± 2.94
Ventriculin	4	21.4 ± 7.26
Methionine	4	17.8 ± 8.20
Yeast	4	7.8 ± 0.86
Liver extract	4	7.2 ± 0.56
Choline	3	7.2 ± 1.08

in preventing these lesions served as a base line to compare the lipotropic effects of the other supplements. Microscopic examination or fat analyses indicated that the liver extract completely prevented the fatty changes in the liver of rats fed a high-fat diet. Supplements of Ventriculin or methionine were without effect, casein was only slightly effective, and yeast was partially effective (TABLE 2). Liver extract was equally potent in preventing fatty livers in animals fed the low-protein diet. With this diet the yeast supplement also offered complete protection whereas Ventriculin and methionine were without lipotropic activity.[5, 6, 7]

Food intake was measured in each study and the lipotropic activity of the supplements was not related to changes in caloric or protein intake. There was also no correlation between lipotropic activity and gain in body weight. In further studies, it was noted that oral liver extract, 1 cc. three times a week is slightly less lipotropic than the same dose given by injection.[8]

The lipotropic activity of these supplements cannot be explained on the basis

Table 3

Lipotropic Effect of Different Vitamin B_{12} Concentrates Prepared From Liver

No. animals	Diet	Dose of conc. in terms of μg. of B_{12}	Liver fat gm.%
	Exp. 1, 66 days		
4	Normal	—	5.6 ± 1.08
8	High fat	—	24.6 ± 0.59
10	High fat	1.0	7.8 ± 0.89
	Exp. 2, 50 days		
6	Normal	—	6.3 ± 0.82
7	High fat	—	15.3 ± 2.01
5	High fat + #4	1.0	13.1 ± 1.01
5	High fat + #4	4.0	13.8 ± 1.27
5	High fat + #2	1.0	11.3 ± 1.35
5	High fat + #2	4.0	10.4 ± 0.95
5	High fat + #3	1.0	7.4 ± 0.88
5	High fat + #3	4.0	7.8 ± 0.62

of their choline content. Of all of the supplements, Ventriculin supplied the largest amount of choline; namely, 1.8 to 2.1 mgm. per day. Although the liver extract completely prevented the fatty changes, it supplied approximately one-third the amount of choline of an ineffective supplement such as Ventriculin. Ventriculin supplied 8 mgm. of methionine per day and liver extract provided only 3 μg. of this amino acid per injection, certainly too small to account for lipotropic effects.

We then observed that certain vitamin B_{12} concentrates prepared from liver were also lipotropic.[9] The activity of such extracts, however, varies quite considerably.[10] The extracts were injected three times a week in terms of their vitamin B_{12} activity and the total amount of extract administered was usually 0.5 cc. three times a week. The lipotropic potency of such extracts can vary from inactivity partial effect to complete prevention of the fatty changes (TABLE 3). These vitamin B_{12} concentrates supplied only 0.2 mg. of choline and 0.08 mg. of methionine per day, an amount too small to explain their lipotropic activity. One concentrate of vitamin B_{12} prepared from streptomyces broth was studied but proved to be too toxic to clearly evaluate lipotropic activity. A growth promoting factor which has been highly purified and called B_{13} is present in Distillers Dried Grain Solubles.[11, 12] This substance affects growth in the rat and chick.[13] Vitamin B_{13} concentrate seems to have a partial lipotropic activity and may merit further study (TABLE 4). The higher dose of the extract, however, was somewhat toxic.[10]

In view of lipotropic effect of liver extract and vitamin B_{12} concentrate, and the fact that their activity could not be explained on the basis of choline content, further studies were designed using the high-fat diet, in which small amounts of supplements were administered in different combinations to rats

Table 4

Effect of Vitamin B_{13} Concentrate on Rats Fed a High Fat Diet for 30 Days

Animals	Diet	B_{13} conc. (3 × week)	Liver fat %
5	Low fat	none	7.02 ± 0.72
5	High fat	none	23.9 ± 1.40
5	High fat	0.3 cc.	18.4 ± 0.24
3*	High fat	0.8 cc.	11.9 ± 4.39

* Two animals died, on the 12th and 26th days.

Table 5

Effect of Small Doses of Single Supplements on Liver Fat

No. animals per group	Normal diet	High-fat diet untreated	High-fat diet treated	Supplement (3 × week)	Dose	Days of exp.
10	4.8 ± 1.20	17.5 ± 1.49	14.1 ± 1.77	B_{12}	1 μg.	30
8–10	6.8 ± 0.31	28.8 ± 1.03	27.3 ± 1.12	B_{12}	2.5 μg.*	64
8–10	8–4 ± 1.05	26.7 ± 1.83	26.2 ± 2.54	B_{12}	1.0 μg.	30
			24.6 ± 0.83	B_{12}	4.0 μg.	30
6	—	18.9 ± 1.98	18.3 ± 2.54	B_{12b}	1.0 μg.	64
10	4.8 ± 1.20	17.5 ± 1.49	23.4 ± 1.83	Choline	1.0 mg.	30
			26.1 ± 2.38	Choline	2.0 mg.	30
6	—	18.9 ± 1.98	16.4 ± 1.27	Folic acid	2.5 mg.	64
			21.0 ± 3.81	Folic acid	25.0 mg.	64
			16.3 ± 2.19	Citrov. fact.	2,500 units	64
			16.8 ± 1.67	Citrov. fact.	25,000 units	64
6	6.3 ± 0.29	24.9 ± 1.14	20.7 ± 1.85	Inositol	2.0 mg.	33
			18.9 ± 2.38	Inositol	4.0 mg.	33
8	5.8 ± 0.27	19.2 ± 1.67	16.4 ± 1.72	Aureomycin	5.0 mg.	31

* 6 times per week

receiving a high-fat diet. The amounts of supplements administered were similar to those present either in liver extract or vitamin B_{12} concentrate. Although small amounts of each material present in liver extract may be ineffective by itself, it may, when given in combination with other materials, demonstrate lipotropic activity. The single supplements studied were B_{12}, B_{12b}, choline, folic acid, citrovorum factor, inositol, and aureomycin. Each of these alone (TABLE 4) in the doses used were ineffective in preventing fatty changes in the liver of animals fed a high-fat diet.[8, 14, 15] György and Rose also reported that crystalline vitamin B_{12} was without lipotropic effect in animals fed a high-fat diet. They did, however, obtain a partial lipotropic effect of vitamin B_{12} in rats receiving a low-fat—low-protein diet.[16]

At this time Shaefer, Salmon, and Strength demonstrated a sparing effect of vitamin B_{12} on choline as judged by the growth of chicks and the prevention of renal hemorrhagic necrosis in rats.[17, 18, 19] Our attempts to demonstrate a sparing effect of B_{12} on choline in the lipotropic studies were unsuccessful (TABLE 5). The doses of choline were graded and were greater than the amount of choline present in liver extract or vitamin B_{12} concentrate.[8] Thus the lipotropic of liver extract cannot be explained by a sparing effect of vitamin B_{12} on the amount of choline present. A combination of choline and vitamin

TABLE 6

EFFECT OF CHOLINE, VITAMIN B_{12}, AND VITAMIN B_{12b} ON LIVER FAT OF RATS FED A HIGH-FAT DIET

No. animals	Diet	Choline mgm.	B_{12} μg.	B_{12b} μg.	Liver fat gm.%
Experiment 1, 30 days					
10	Normal	—	—	—	4.8 ± 1.20
10	High-fat	—	—	—	17.5 ± 1.49
9	High-fat	0.5	1.0	—	19.0 ± 1.53
10	High-fat	1.0	1.0	—	23.5 ± 1.26
10	High-fat	2.0	1.0	—	18.9 ± 2.24
Experiment 2, 64 days					
6	High-fat	—	—	—	18.9 ± 1.98
5	High-fat	2.0	—	1.0	20.1 ± 2.27
Experiment 3, 72 days					
4	Normal	—	—	—	5.2 ± 0.75
5	High-fat	—	—	—	23.7 ± 1.72
6	High-fat	4	2	—	19.2 ± 0.57

TABLE 7

LIPOTROPIC EFFECT OF CHOLINE, VITAMIN B_{12} AND B_{12b} IN COMBINATION WITH FOLIC ACID AND CITROVORUM FACTOR

No. animals	Diet	Choline mg.	B_{12} μg.	B_{12b} μg.	Folic acid μg.	Citrov. factor units	Liver fat gm.%
Experiment 1, 64 days							
6	High fat	—	—	—	—	—	18.9 ± 1.98
6	High fat	2	1	—	—	2,500	16.9 ± 1.08
6	High fat	2	1	—	—	25,000	11.4 ± 1.71
6	High fat	2	1	—	2.5	—	12.1 ± 0.65
6	High fat	2	1	—	25.0	—	11.4 ± 0.96
5	High fat	2	—	1	—	25,000	10.7 ± 1.48
5	High fat	2	—	1	25.0	—	10.5 ± 1.12
Experiment 2, 72 days							
4	Normal	—	—	—	—	—	5.2 ± 0.75
5	High fat	—	—	—	—	—	23.7 ± 1.72
6	High fat	2	1	—	2.5	—	14.4 ± 1.12
7	High fat	4	2	—	2.5	—	14.3 ± 0.78

B_{12b} was also ineffective[14] and vitamin B_{12} does not exert a sparing effect on methionine in lipotropic studies[16] (TABLE 6).

During the past two years studies were also performed with folic acid or citrovorum factor in combination with choline and vitamin B_{12}. In the previous studies, the liver extract supplied an average 1.2 μg. and the vitamin B_{12} concentrate an average of 0.03 μg. of folic acid per day. The addition of

TABLE 8

LIPOTROPIC EFFECT OF CHOLINE, VITAMIN B_{12}, FOLIC ACID AND INOSITOL

No. animals	Diet	Choline mgm.	B_{12} µg.	Folic acid µg.	Inositol mg.	Liver fat gm.%
Experiment 1, 92 Days						
12	Normal	—	—	—	—	6.8 ± 0.49
12	High-fat	—	—	—	—	18.7 ± 1.39
9	High-fat	0.5	—	1	0.5	20.1 ± 0.58
10	High-fat	1.0	—	2	1.0	17.7 ± 2.48
Experiment 2, 33 Days						
6	Normal	—	—	—	—	6.3 ± 0.29
6	High-fat	—	—	—	—	24.9 ± 1.14
6	High-fat	2.0	—	2.5	2.0	19.5 ± 1.10
6	High-fat	2.0	—	10.0	2.0	17.3 ± 3.25
6	High-fat	2.0	1.0	2.5	2.0	14.9 ± 1.62
5	High-fat	2.0	1.0	10.0	2.0	16.8 ± 2.30
6	High-fat	2.0	1.0	10.0	4.0	15.8 ± 2.10

TABLE 9

LIPOTROPIC EFFECT OF SMALL AMOUNTS OF VARIOUS SUBSTANCES

No. animals	Diet	Choline mg.	B_{12} µg.	Folic acid µg.	Inositol mg.	Aureomycin mg.	Liver fat gm.%
6	Normal	—	—	—	—	—	5.8 ± 0.27
8	High-fat	—	—	—	—	—	19.2 ± 1.67
8	High-fat	2	—	10	4	5	17.6 ± 2.05
9	High-fat	2	1	10	—	5	11.0 ± 0.83
8	High-fat	2	1	10	4	5	12.6 ± 1.71

folic acid to the choline and the B_{12} produced a partial lipotropic effect.[14] This effect of folic acid could also be obtained by administering 25,000 units of citrovorum factor with the B_{12} and choline, and vitamin B_{12b} was also effective in replacing vitamin B_{12} in such a combination of supplements (TABLE 7). These three supplements were not as effective as liver extract, for in each study the liver fat was only returned about half-way to normal.

In the earlier studies, the liver extract supplied 0.9 mg. of inositol per injection and the yeast 1.1 mg. of inositol per day. The addition of inositol to the other supplements, however, did not add to the partial lipotropic effect of the mixture (TABLE 8). In the last study, aureomycin was included but without further effect on lipotropic activity (TABLE 9).[15]

Discussion

The establishment in 1948 of the lipotropic activity of liver extract in rats fed a high-fat diet demonstrated that factors other than choline can markedly influence the fat content of the liver.[6] The lipotropic effects of vitamin B_{12} concentrates prepared from liver are also not explainable on the basis of choline content.[9, 10] That vitamin B_{12} was important was demonstrated by Schaefer and co-workers who reported a sparing action of this vitamin on the choline requirement of rats and chicks.[17, 18] Vitamin B_{12} and other factors may act to alter either the rate of synthesis of labile methyl groups or the process of transmethylation, but the exact mechanism of such effects is not known. It is now known that the methyl groups of choline and methionine can be synthesized from substances such as formate, acetate, L-serine, and glycine.[20, 21, 22, 23] Bennett reported in 1949 and 1950 that rats can grow on a diet free of methyl group donors provided vitamin B_{12}, folic acid, and hemocystine are supplied,[24] and Stekol and Weiss obtained similar results.[25] Schaefer and Knowles, however, concluded that supplements of vitamin B_{12}, folacin, and homocystine did not prevent the renal injury and death of weanling rats (40–50 gm.) in the absence of a dietary source of labile methyl groups.[26] The variation in results may be due to the difference in requirement among the strains of rats.[27, 28]

Other studies have also shown that vitamin B_{12} is necessary for the methylation of homocystine to form methionine. A diet deficient in vitamin B_{12} interferes with the ability of the liver to convert betaine and homocystine to methionine or choline and homocystine to methionine.[28] Jukes, Stokstad, and Broquist observed that vitamin B_{12} was necessary for the methylation of homocystine to obtain growth in chicks fed a methionine-deficient diet.[30, 31] This action of vitamin B_{12} depends, however, on the amount of methyl groups available. When weanling rats are fed a diet deficient in choline and methionine, it was observed that choline alone will prevent the renal injury but gain in body weight is very slight. In the presence of choline, Schaefer and Knowles[26] observed that supplements of vitamin B_{12} alone allowed an adequate gain in weight, indicating that the synthesis of methionine from homocystine was facilitated. When, however, the amount of methyl groups was limited by a further reduction of choline intake, both vitamin B_{12} and folacin were required to allow adequate growth.

A deficiency of folic acid in the chicken has also been reported to interfere with the methylation of homocystine,[31] and still other studies show the need for both folic acid and vitamin B_{12}. Schaefer and Knowles[26] showed that supplementations of a basal diet with homocystine, betaine, and aminoethanol failed to support growth or prevent renal injury. With 0.41 per cent betaine and 0.05 per cent aminoethanol in the diet, the addition of vitamin B_{12} prevented renal injury and permitted good growth. With a lesser amount of betain and aminoethanol in the diet, supplements of both vitamin B_{12} and folic acid were required to prevent renal injury and allow growth. Such weanling rats fed deficient diets also develop fatty livers. The addition of vitamin B_{12} and folic acid suppressed the accumulation of fat in the liver of rats fed subprotective levels of choline.[28, 32]

Of particular interest is the relationship of folic acid to choline oxidase and betaine aldehyde oxidase which are necessary for transmethylation reactions from choline *in vitro*. It has been reported by various groups that aminopterin, a folic acid antagonist, inhibits choline oxidase activity.[33, 34, 35] Williams[36] has shown that aminopterin not only depresses choline oxidase activity but also betaine aldehyde oxidase and the aerobic and anaerobic transmethylation to homocystine.[37] It would appear then that folic acid plays a part in the conversion of choline or betaine aldehyde to the actual methyl donor, betaine, and its transmethylation to homocystine. Stekol *et al.* have found that a deficiency of folic acid in the adult rat reduces the capacity to utilize glycine or serine for the production of choline. During vitamin B_{12} deficiency, only the utilization of glycine is reduced.[38]

How do the above data compare with the results obtained in studies designed primarily to induce liver injury? In our own studies, we have generally utilized the high-fat diet. In an attempt to explain the lipotropic effect of liver extract, the various substances present in liver extract were administered alone and in various combinations. The amount of each supplement was equal to or slightly greater than that present in liver extract or vitamin B_{12} concentrates. The small amounts of choline or vitamin B_{12} present in such liver extract were without lipotropic effect and no sparing effect could be demonstrated when the vitamin B_{12} and choline were administered together. It is probable, under the conditions of these studies, that the requirements for choline are too great to enable a sparing effect of vitamin B_{12} to be demonstrated. The further addition of folic acid to a combination of vitamin B_{12} and choline produced a partial reduction in liver fat. The amount of folic acid used was about ten times that present in liver extract. The folic acid was not effective alone and had to be used in combination with choline and vitamin B_{12}. The further addition of inositol or aureomycin failed to influence further the deposition of fat in the liver. The best results, therefore, have been obtained with a mixture of these three supplements and there must be other factors in the liver extract that can influence the synthesis of labile methyl groups.

With regard to other diets and results, György and co-workers reported that liver extract may enhance the effect of casein or of methionine in treating experimental cirrhosis[39] and that vitamin B_{12} does not affect the production of necrosis with a necrogenic diet.[16] Using normal diets, Popper[40] has also found that vitamin B_{12} concentrate will inhibit the fatty changes and depletion of ribonucleic acid in the liver of rats receiving a single injection of carbon tetrachloride. This was confirmed by Mushett[41] using crystalline vitamin B_{12}. The vitamin B_{12} was without effect on the liver when repeated doses of carbon tetrachloride were given. The continuation of vitamin B_{12} treatment after cessation of carbon tetrachloride, however, brought about a more rapid reversal of the hepatic damage. Hove has also demonstrated that vitamin E or vitamin B_{12} can increase the survival, under certain dietary conditions, of rats receiving toxic doses of CCl_4.[42] The choline oxidase activity of the liver was not influenced by vitamin E or by CCl_4.[43]

Aureomycin may exert a sparing effect on vitamin B_{12}, as measured by growth response in rats and chickens,[44, 45, 46] and György has reported that aureomycin can exert a lipotropic effect and an antinecrotic effect in experimental animals.[47, 48] It is generally assumed that aureomycin given orally acts to alter the bacterial floor of the gut. This may occur by a decrease in the number of *E. coli* in the gut and a simultaneous increase in *Bacillus megatherium,* an organism that can produce large quantities of vitamin B_{12}.[49] In our own studies, the administration of aureomycin by injection did not improve the partial lipotropic effect of folic acid, vitamin B_{12}, and choline.

The problem becomes more complicated by interrelationship between cortisone, vitamin B_{12}, and folic acid. Cortisone will increase the excretion of vitamin B_{12} in pigs fed a vitamin B_{12}-deficient diet and thereby reduce body growth and survival.[50] Vitamin B_{12} or aureomycin can largely counteract the growth-inhibiting effects of cortisone in rats.[51] Cortisone can also replace pteroylglutamic acid and citrovorum factor for the growth of *Streptococcus faecalis* and *Leuconostoc citrovorum* and can increase the growth-inhibiting effects of aminopterin on *Leuconostoc citrovorum*.[52]

Summary

The lipotropic effect of liver extract or certain vitamin B_{12} concentrates prepared from liver cannot be accounted for by their content of choline. Using various supplements in the amounts present in these extracts, it was found that a combination of choline, vitamin B_{12}, and folic acid produced a partial lowering of liver fat in rats fed a high-fat diet. In the absence of any one supplement from this mixture, liver fat was not even partially reduced. Vitamin B_{12} could be replaced by vitamin B_{12b} and citrovorum factor could replace folic acid. The further addition of inositol and aureomycin was without added effect in lowering liver fat.

Investigators have shown a certain relationship of vitamin B_{12} and folic acid to the synthesis of labile methyl groups and to their use in the transmethylation procedures, as judged by the growth of animals or the prevention of renal injury. It is evident that, when diets are primarily designed to produce fatty changes in the liver, a factor or factors other than choline, vitamin B_{12}, and folic acid are required to allow maintenance of a normal liver fat.

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Discussion of the Paper

Doctor Schwarz: In the introduction I voiced the opinion that there exist unidentified factors against fatty liver and probably also against cirrhosis. I am glad to see that Doctor Drill in his very careful studies has obtained evidence which has led him to the same conclusion. In our work on the concentration and purification of Factor 3 from natural sources, especially from enzymatic casein digests, we have found that certain fractions do *not* protect against dietary necrotic liver degeneration, but actually *enhance* development of the disease. We have accumulated much evidence that sources of methyl groups such as choline and betaine, and also substances which are catalytically involved in one-carbon-unit metabolism, such as vitamin B_{12} and citrovorum factor, have such an enhancing effect on the development of liver necrosis (Schwarz, Introduction, page 617). According to our conception that there is an antagonistic balance between liver necrosis on the one hand, and fatty liver and cirrhosis on the other hand, we have tested our liver-necrosis-accelerating fractions for protective activity against fatty infiltration of the liver. In preliminary tests it was found that they depress the amount of fat accumulated in the rat liver on choline-vitamin B_{12}-free diets. This lipotropic activity is *not* due to the presence of either B_{12} or choline, but must be due to other unidentified constituents, the exact nature of which awaits further elucidation.

LIVER CARCINOMA AND RELATED LESIONS IN CHRONIC CHOLINE DEFICIENCY*

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In studies of the pathology of chronic choline deficiency in the rat, the development of various types of neoplasms was observed in a significant percentage of experimental animals. Some of these results have been reported by Copeland and Salmon in 1946,[1] Engel, Copeland, and Salmon in 1947,[2] and Schaefer, Copeland, Salmon, and Hale in 1950.[3] Over 500 chronic choline-deficient rats have been studied in this laboratory. Since the finding of neoplasms in these animals was most frequent in the liver, it is the purpose of this paper to describe the sequence of lesions culminating in the appearance of liver carcinoma. In another series of experiments, chickens were used to determine whether it would be possible to produce neoplasms by chronic choline deficiency in a species of animal other than the rat. The fatty changes, cirrhosis, and neoplasms of the liver, as well as some kidney changes which developed, are compared to corresponding lesions in the rat.

The general procedure followed and typical choline-deficient diets used in our laboratory have been described in previous publications.[1, 4]

Histologic Studies on Livers of Chronic Choline-Deficient Rats

Fatty livers and cirrhosis. The livers of rats that died during the first four weeks of the experiments were swollen and quite light in color. Microscopically there was a marked accumulation of fat uniformly involving all lobes of the liver, and apparently all the liver cells. On the other hand, sections from the livers of rats that had been on the choline-deficient diet for as long as three months showed a zonal distribution of fatty infiltration with marked accumulations of fat in some areas forming peculiar architectural alterations.[1] These are similar to lesions described as "fatty cysts" by Hartroft and Ridout.[5] In later stages of the deficiency, the amount of fat appeared to decrease as cirrhosis advanced in extent and severity. Some lobules of the liver contained considerable fat but others seemed to be composed largely of newly regenerated liver cells. Ceroid was present in all cirrhotic livers but varied in amount. Mitotic figures were found in the cirrhotic livers indicating that regeneration of new parenchymal cells was taking place at a rapid rate. There was a pronounced variation in the size of the liver cells. Bile duct proliferation was seen in a majority of the livers.

Neoplasms of liver. In the livers of all the rats that remained on the choline-deficient diets for eight months or longer, severe nodular cirrhosis was observed. Large regeneration nodules 0.5 to 1 cm. in diameter were common. Tumors

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* Published with the approval of the Director of the Alabama Agricultural Experiment Station. This work was supported in part by grants from the American Cancer Society upon the recommendation of the Committee on Growth of the National Research Council, from the National Cancer Institute, and from the Nutrition Foundation, Inc.

The authors are also indebted to Dr. William V. Hare, Pathology Department, University of Mississippi Medical School, for his assistance in interpretation of the microscopic material.

developed in a significant number of cases. The incidence of tumors of the liver in the rat varied in the different experiments from 16.1 to 30 per cent with an average of 23.1 per cent. The tumors varied in size from 0.8 to 3.5 cm. in diameter and were of a different consistency from the regeneration nodules. The gross appearance of one of the liver tumors is illustrated in FIGURE 1. The histopathology of this type of tumor is illustrated in FIGURES 2, 3, and 4. It is apparent from this series of photomicrographs that there is a variation in the cells and arrangement of cells that compose an individual tumor. The alveolar arrangement and general appearance of the cells in some areas seem to point to a bile duct origin, but in most cases the cell type suggests an origin from liver cord cells.

Histologic studies are being continued on all tumors that developed in chronic choline-deficient rats in an attempt to determine which of these tumors are definitely malignant. Special stains were carried out on most malignant or "possibly malignant" lesions. Histologic determination of the malignancy of many of these "tumors" is a matter of judgment. We have attempted to be conservative by grouping many of them under the classification "atypical regeneration nodules."

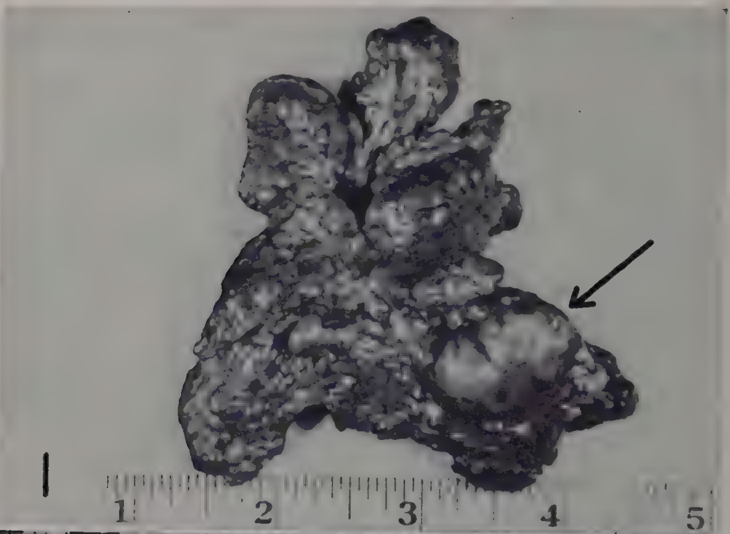
In many tumor cases, invasion of the mesentery was noted grossly and verified microscopically. In some of these cases, little or no connection was noted between the liver proper and the outlying nodules. Microscopically, some of these tumor islands appeared to be lying in lymph vessels or dilated venous channels. A fibrous wall was generally found around them. Slides prepared with Verhoeff's elastic tissue stain and by the periodic acid method did not prove that these structures were blood vessels. However, this did not eliminate the possibility of these structures representing lymphatic invasion. The appearance of the tumor nodules in these areas suggests that, as the tumors spread into mesenteric tissue, they incite the production of a limiting fibrous wall. One slide in each of two cases did show definite vein invasion. With the exception of these two cases, the stroma of the tumor blended too well with the fibrous wall for that wall to be a blood vessel. There were proliferating bile ducts at the edge of some of the tumor islands.

An interesting feature in the study of periodic acid stains on sections of the liver tumors is that there is a large amount of glycogen present in the cirrhotic, nontumorous portions of the livers, but there is practically none in the "atypical regeneration nodules" or tumor tissue proper.

Slides stained with Mallory's connective tissue stain showed that there was a large amount of collagen in the cirrhotic, nontumorous portions of the liver; but in the tumor proper, there was a relatively small amount around alveolar structures. In slides prepared by Foot's modification of Bielschowsky's reticulum stain, there is a characteristic alveolar pattern in some parts of the tumor, which is typical of epithelial origin.

No fibrils arising from tumor cells were demonstrated by phosphotungstic acid stain. There was some PAS positive material in some of the macrophages in the cirrhotic livers which is probably ceroid.

All the blocks of lung tissue from choline-deficient rats with liver tumors are



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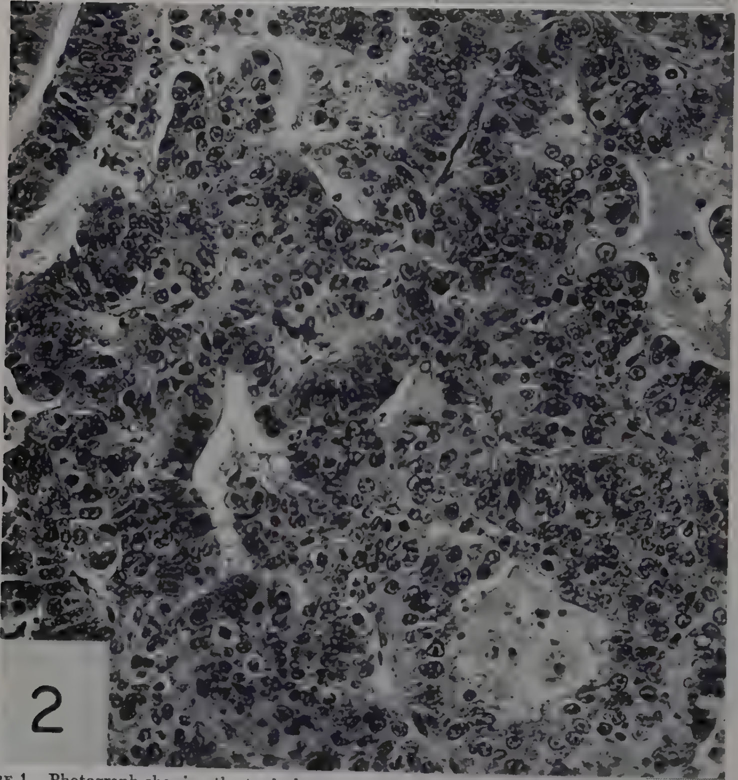

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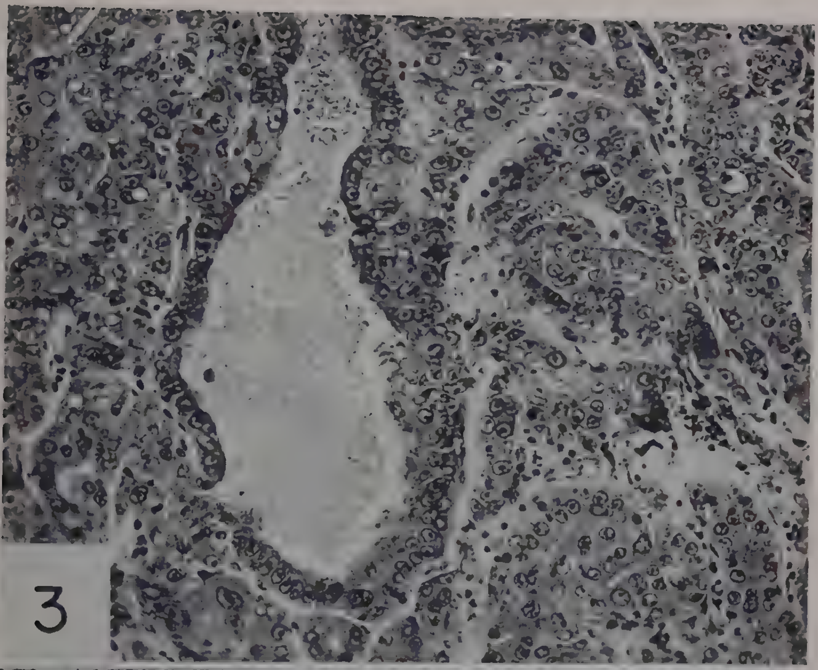
Figure 1. Photograph showing the typical gross appearance of a liver tumor from a choline-deficient rat. Reduced to three-fourths actual size.

Figure 2. Photomicrograph of a section of the liver tumor illustrated in figure 1. In this area the tumor cells form "ductlike" structures. Hematoxylin and eosin stain. ×230.



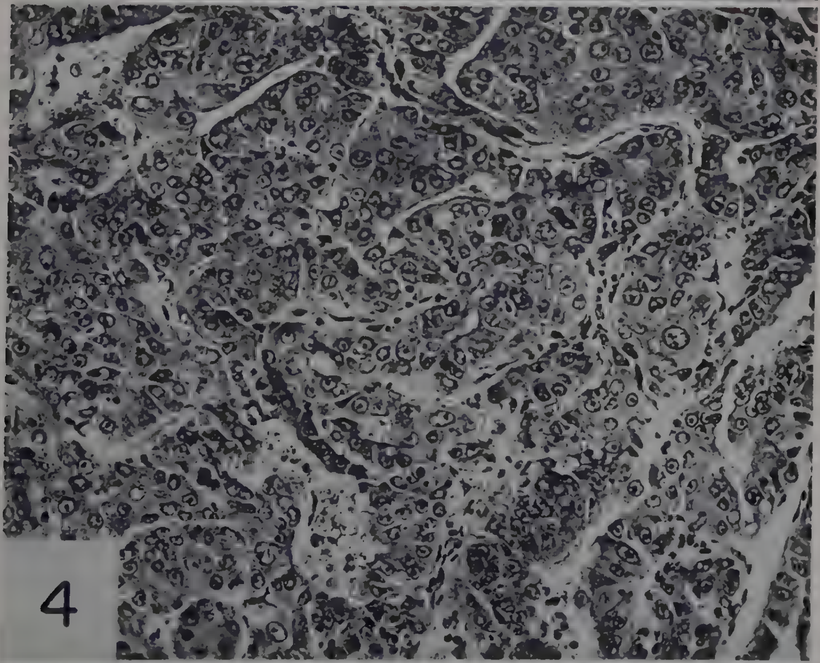


FIGURE 3. Photomicrograph of a section of another part of the tumor illustrated in FIGURE 1. The tumor cells are forming "ductlike" structures, but there is a transition to areas with more closely packed cells. Hematoxylin and eosin stain. ×230.

FIGURE 4. Photomicrograph of a section of a liver tumor from a choline-deficient rat. The arrangement of the cells differs from that shown in FIGURES 2 and 3. Hematoxylin and eosin stain. ×230.

being recut. In the process of this study, two cases of pulmonary metastases from liver tumors have been found. In each case, an embolus was found in a blood vessel in the lung in addition to the metastatic nodule of tumor tissue. A photomicrograph of an embolus of tumor tissue (from liver tumor) in a blood vessel in the lung of a choline-deficient rat (no. 14065) is shown in FIGURE 5. The pulmonary metastasis from this liver tumor is illustrated in FIGURE 6. A pulmonary blood vessel containing an embolus of tumor cells (rat no. 14067) is illustrated in FIGURE 7. The pulmonary metastasis from a liver tumor in this rat is shown in FIGURE 8.

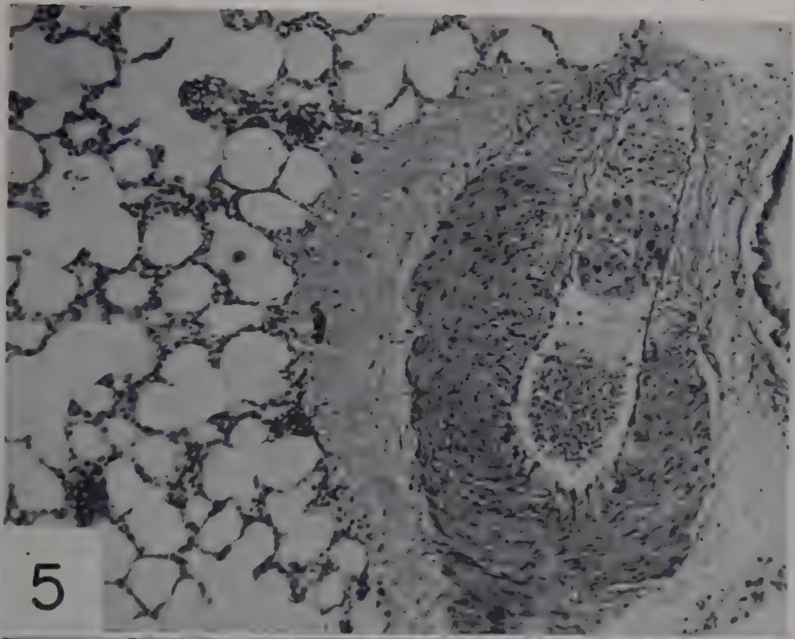
Although only two cases of pulmonary metastases from liver tumors have been found to date in our choline-deficient rats, this serves to prove that at least some of these tumors are malignant. The microscopic appearance of the two metastasizing tumors did not differ materially from many of those in which no metastases were found.

Histopathology of the Livers of Chronic Choline-Deficient Chickens

Microscopic studies of slides prepared from the tissues of 105 chronic choline-deficient chickens and 33 control chickens fed the same diets supplemented with 0.3 to 0.6 per cent choline chloride have been made. The chickens remained on the choline-deficient diet 28 to 133 weeks (average 78 weeks). The control chickens remained on the diet for 44 to 133 weeks (average of 80 weeks). Of the 105 chickens, 38 developed neoplasms of various kinds, which were multiple in 12 chickens. No neoplasm was found in the 33 control chickens.

Fatty liver and cirrhosis. There was considerable variation in the gross appearance of the livers of the chronic choline-deficient chickens. The livers varied in size, the majority of them being very large and fatty. An interesting feature of the fatty livers was that the entire liver was usually not involved. Scattered areas on each lobe frequently were of a normal, dark-red color, the remainder of the liver having a light colored, fatty appearance. This was confirmed by microscopic studies of slides which showed some areas of normal liver tissue immediately adjacent to, but sharply demarcated from, extremely fatty areas (FIGURE 9). The normal areas may represent regenerated liver tissue although they had the appearance of unchanged hepatic parenchyma. Gross nodular cirrhosis so frequently found in the livers of choline deficient rats was observed in only two cases, but frequently was observed microscopically (FIGURE 10).

Neoplasms of liver. Lesions resembling tumors were found in 23 cases. Eleven of these were classified as atypical regeneration nodules, 10 as cholangiocarcinoma, and 2 as hemangioendothelioma. The gross appearance of one of the liver tumors is illustrated in FIGURE 11. The tumors varied in size from 0.8 to 3 cm. in diameter. The microscopic appearance of the liver tumors is illustrated in FIGURES 12, 13, and 14. There was a difference in the arrangement of the cells in different parts of the tumors varying from closely packed cells without any apparent order to well-formed glandular and ductal structures. In some cases, the neoplastic cells closely resembled liver cells; but in most cases, they were anaplastic. FIGURE 12 illustrates one of the tumors in which



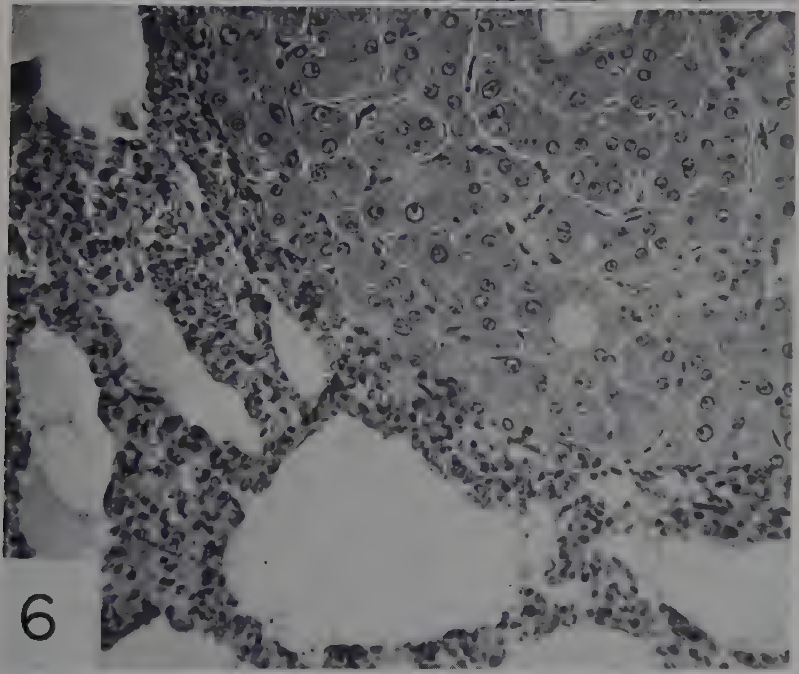
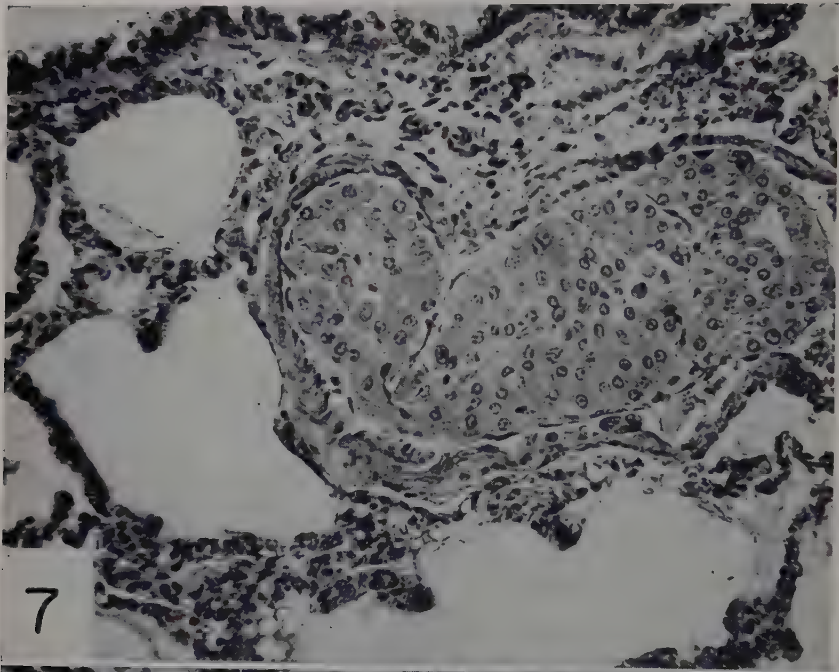


FIGURE 5. Photomicrograph of a blood vessel in the lung of choline-deficient rat no. 14065 containing an embolus of tumor cells from a hepatocellular carcinoma of the liver. Hematoxylin and eosin. ×150.

FIGURE 6. Photomicrograph of a pulmonary metastasis from a liver tumor in choline-deficient rat no. 14065. Hematoxylin and eosin. ×310.



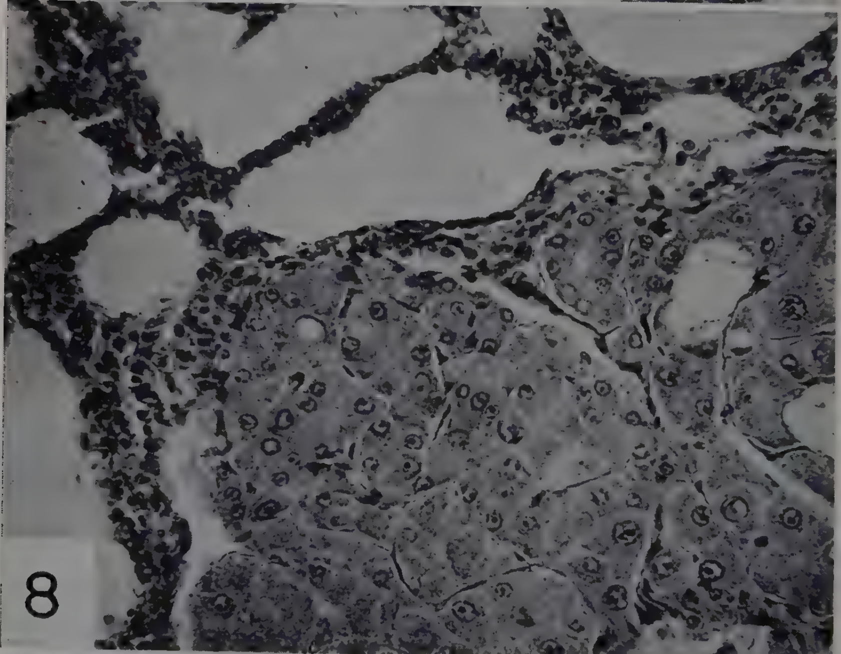
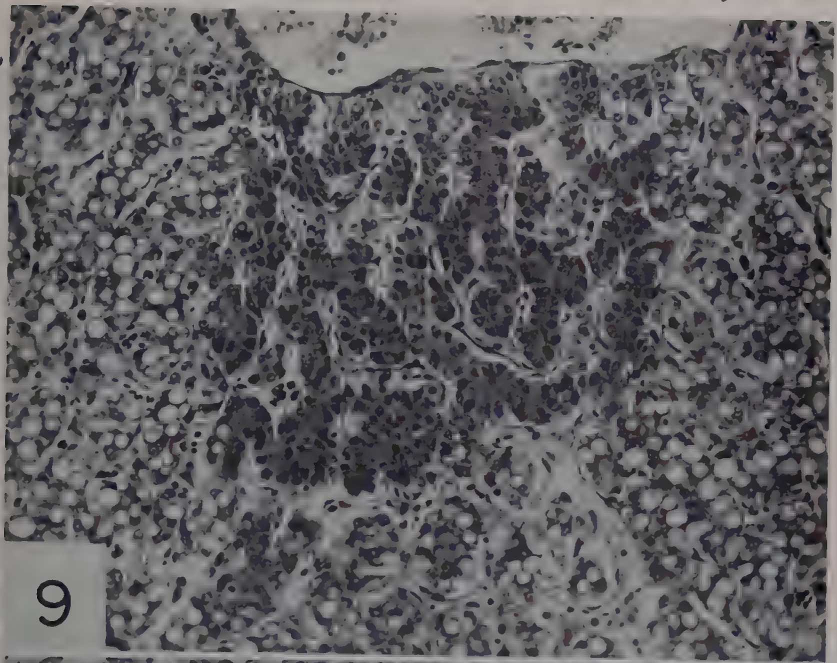


FIGURE 7. Photomicrograph of an embolus of tumor cells in a blood vessel in the lung of choline-deficient rat no. 14067. Invasion from liver tumor. Hematoxylin and eosin. ×275.

FIGURE 8. Photomicrograph of a pulmonary metastasis from a liver tumor in choline-deficient rat no. 14067. Hematoxylin and eosin. ×310.



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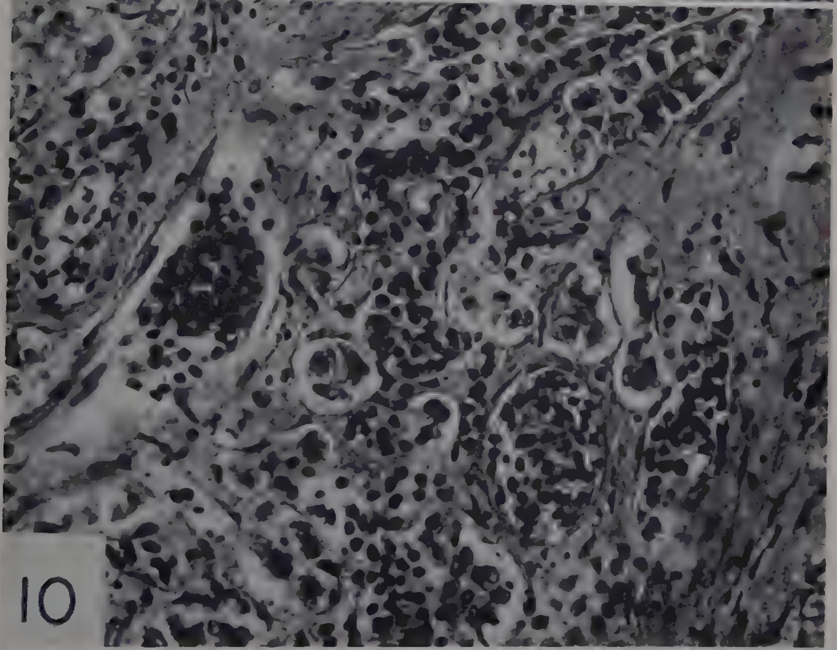

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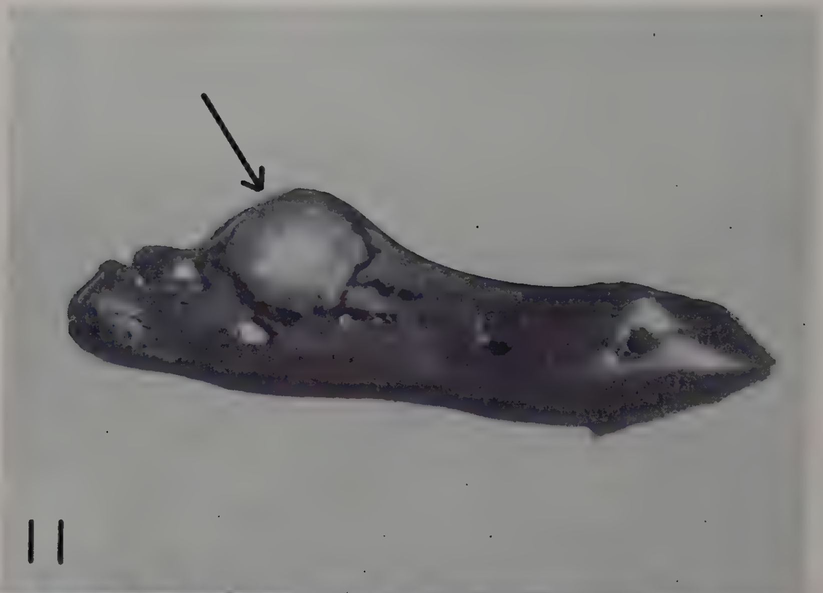
FIGURE 9. Photomicrograph of a section of liver from chicken 717 to illustrate extremely fatty conditions surrounding areas in which the parenchyma is normal. This may represent regenerated liver tissue. Hematoxylin and eosin. ×240.

FIGURE 10. Photomicrograph of liver from chicken 211. This illustrates one of the cirrhotic areas which were found in the livers of chronic choline-deficient chickens. Hematoxylin and eosin. ×450.



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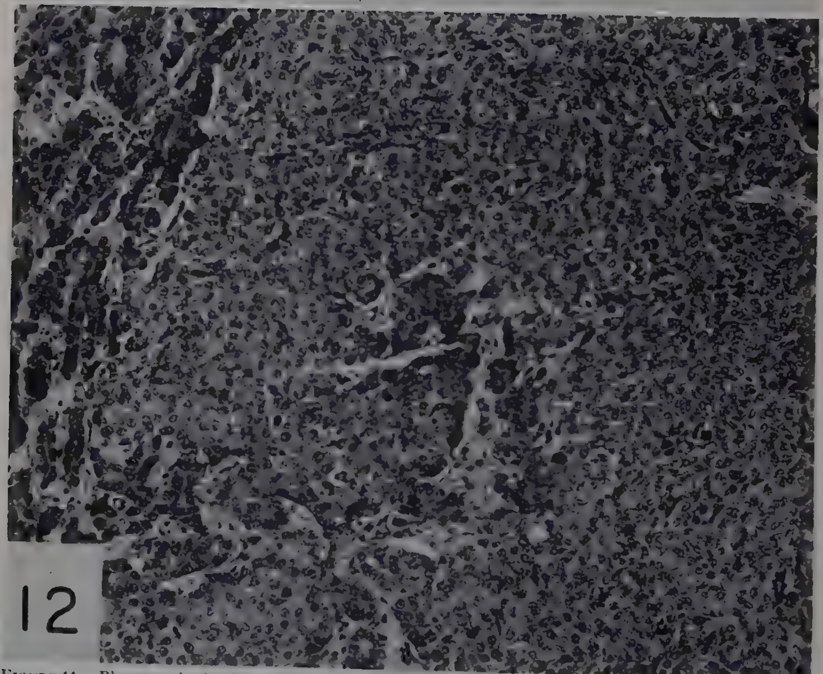

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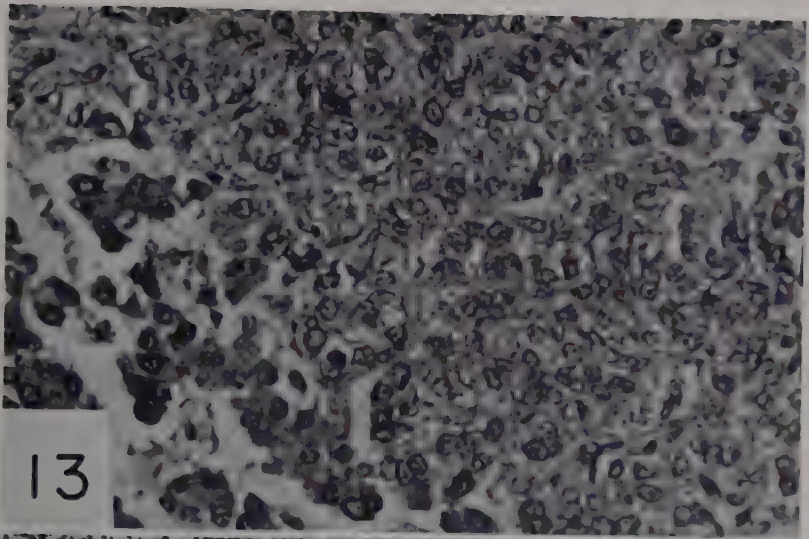
FIGURE 11. Photograph of a slice of liver of a choline-deficient chicken to illustrate the gross appearance of one of the tumors. Natural size.

FIGURE 12. Photomicrograph of a section through carcinoma of the liver of the chicken no. 729 which illustrates the relationship of the tumor to liver parenchyma. Hematoxylin and eosin. ×220.



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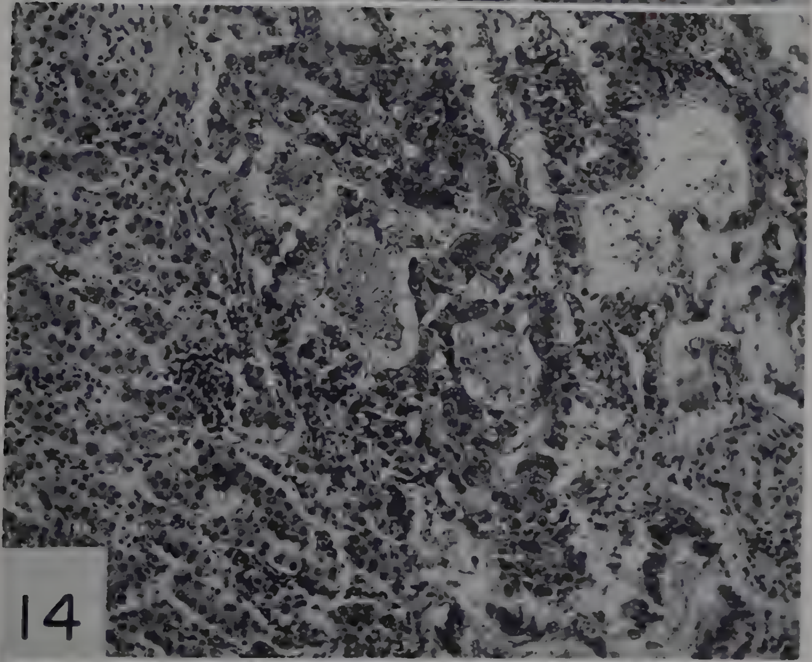

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Figure 13. Photomicrograph of an area of a tumor of the liver of a choline deficient chicken, enlarged to show the nature of the cells. Hematoxylin and eosin. ×400.

Figure 14. Photomicrograph of a section from a tumor of the liver of a choline deficient chicken. The cells form "ductlike" structures. There is some normal liver adjacent to the tumor. Hematoxylin and eosin. ×220.

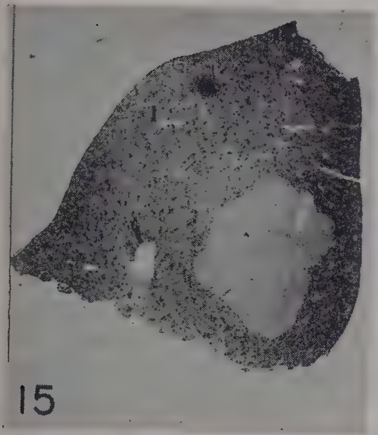
the cells are so compactly arranged that cytoplasmic borders are not visible. The cytoplasm of the tumor cells does not stain as dark as the adjacent liver cells. The nuclei have irregular shapes and contain one or two nucleoli. In all the tumors, there were areas in which the cells were arranged in alveolar fashion with small and large ductlike structures. Periodic acid stain revealed that the tumor cells, in general, tended to contain less glycogen than the surrounding liver tissue. Some of the larger ductlike structures contained considerable amounts of pink staining material, probably mucus, thus being compatible with ductal origin of the tumor. Mucicarmine stains demonstrated only a small amount of mucus. Mucus was not demonstrable in atypical regeneration nodules or carcinomas with closely packed cells. In slides prepared by Foot's modification of Bielschowsky's reticulum stain, there was a heavy reticulum network throughout these tumors with a characteristic alveolar pattern. The reticulum fibers occasionally extended between individual cells in the epithelial island, but characteristically did not. No fibroglia were demonstrable in the sections of liver tumors stained with phosphotungstic acid hematoxylin.

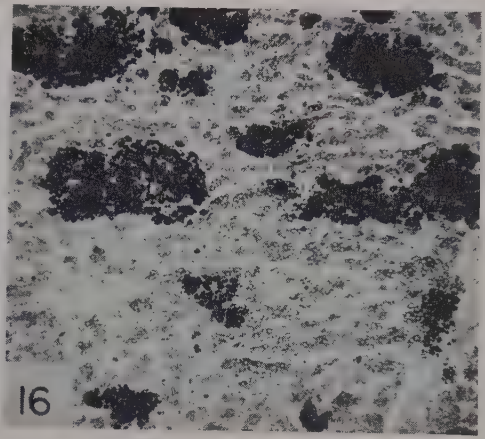
Increase in Iron-Positive Pigment in Liver and Kidney of Chronic Choline-Deficient Chickens

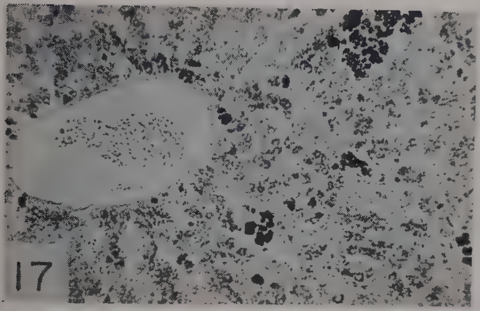
In studies of the microscopic appearance of tissues of chronic choline-deficient chickens, an excessive deposition of pigment was observed in the livers and kidneys. Large accumulations of intracellular and extracellular pigment were observed in the livers, but in the kidneys the pigment was mostly intracellular. The intracellular hepatic pigment was occasionally seen in histiocytes, but primarily in epithelial cells. In sections stained with hematoxylin and eosin, the pigment was found in the form of yellowish-brown granules, varying slightly in size and depth of color and occasionally appearing as conglomerate masses.

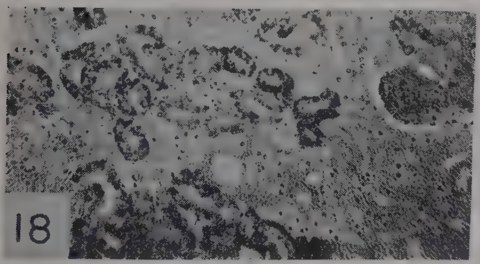
Iron stains. The pigment gave a positive reaction for iron (Turnbull's blue). A photograph of an entire section of liver illustrating the positive iron reaction is shown in FIGURE 15. It is of interest to note that the atypical regeneration nodule in the center of this section did not contain pigment, but there was a large amount distributed throughout the rest of the liver. A similar distribution was observed in the case of the livers that contained tumors. A slide of liver containing a large amount of intracellular and extracellular pigment is shown in FIGURE 16. The pigment in the epithelial cells is in the form of fine granules, but the extracellular deposits are large and small aggregates of granules. The general distribution of this pigment is illustrated in FIGURE 17. A photomicrograph illustrating the deposition of iron positive pigment in the convoluted tubules of the kidney is shown in FIGURE 18. A slide illustrating the difference in the amount of pigment in the convoluted tubules and collecting ducts of the kidney is shown in FIGURE 19.

The pigment gave the positive stain reactions for iron and was, therefore, identified as hemosiderin. The possibility that it might be the acid formaldehyde hematin ("formol") pigment described by Lillie[6] was considered, but the positive reaction with the ferrocyanides rules out this possibility. This possi-









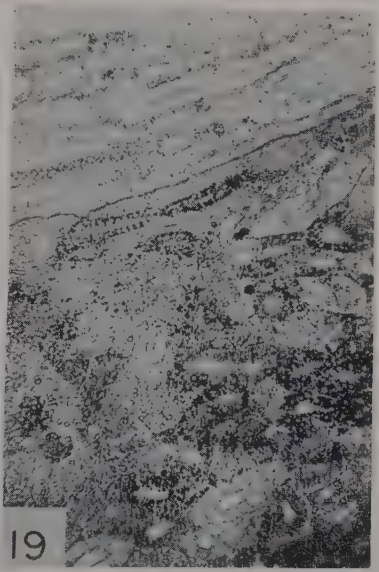


FIGURE 15. Photograph of a slide showing an entire section of liver from a choline-deficient chicken. The atypical regeneration nodule gives a negative iron reaction but the rest of the section gives a positive iron reaction. Iron stain. ×2.

FIGURE 16. Photomicrograph of a section of liver of a choline-deficient chicken illustrating positive iron reaction. There are intracellular and extracellular deposits. Iron stain. ×200.

FIGURE 17. Photomicrograph of a section of liver of a choline-deficient chicken showing general distribution of the iron-positive pigment. Iron stain. ×180.

FIGURE 18. Photomicrograph of a section of kidney of a choline-deficient chicken illustrating the positive iron reaction in the cells of the convoluted tubules. Iron stain. ×180.

FIGURE 19. Photomicrograph of a section of kidney of a choline-deficient chicken to illustrate the difference in amount of pigment in the convoluted tubules and collecting ducts (upper portion). Iron stain. ×180.

bility was further eliminated by the fact that tissues fixed by three different fixatives gave the same reaction.

Discussion

The manifestations of choline deficiency in the chicken were somewhat different from those of the rat under the conditions of our experiments. In contrast to the uniform accumulations of fat in the livers of rats in early stages, the chicken livers were not uniformly fatty. In some cases, entire lobes of a chicken liver contained very little fat even microscopically. This feature may account for the fact that nodular cirrhosis was found to involve the entire liver in only two cases, and that, in the majority of cases, only scattered areas of the livers were cirrhotic.

A definite hemochromatosis (defined as hepatic cirrhosis with iron deposits in the liver and other organs) developed in approximately 25 per cent of the choline-deficient chickens, while more than normal quantities of iron-positive pigment were found in the livers and kidneys of nearly all the birds. Slides of the liver and kidney from control chickens fed the same diet, but with choline added, were carefully checked for the presence of the pigment. There was a small amount present in some of the older chickens. The amount of pigment in the controls was extremely small in comparison to the large amount in the deficient chickens. In contrast to the excessive pigmentation of the livers and kidneys of chronic choline-deficient chickens, the livers and kidneys of choline-deficient rats showed only moderate amounts of the pigments. The livers and kidneys of the choline-deficient rats, however. did show a definite increase in iron-positive pigments over the livers and kidneys of the control rats. Only minute traces of the pigment could be seen in livers and kidneys of the control rats.

Warren and Drake[7] have discussed the relationship between hemochromatosis and primary carcinoma of the liver in man and have published a pertinent bibliography. Gillman, Mandelstam, and Gillman[8] have described the presence of large amounts of iron pigment in human cirrhotic livers in Africans whose diets appeared to be inadequate in quality.

It should be pointed out that the diets of the rats and chickens were free of vitamin B_{12} and that the rat diets were also deficient in folic acid. It has been shown in this laboratory that vitamin B_{12} has a definite choline-sparing action.[9, 10, 11] We have not found tumors in the livers of rats receiving choline-deficient diets supplemented with vitamin B_{12}.

Summary

(1) Studies have been carried out on chronic choline-deficient rats and chickens. The most prominent lesions have developed in the livers and kidneys of these animals.

(2) In the rat liver, fatty infiltration has developed consistently and has been followed eventually in a large number of cases by cirrhosis and, in some cases, by tumor formation. Pulmonary metastases from liver tumors were found in two rats.

(3) In the chicken liver, focal rather than generalized reactions were usually observed. Fatty changes and cirrhosis were common. Lesions considered to be hepatic malignancies developed in 12 cases, with 11 others showing "atypical regeneration nodules."

(4) Increased pigmentation with an iron-positive pigment (hemosiderin) was observed in the livers and spleens of the choline-deficient rats and chickens, but was much more severe in the chickens.

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HEPATOMAS PRODUCED IN MICE BY FEEDING BENTONITE IN THE DIET

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It is the purpose of this paper to report the occurrence of hepatomas in mice fed a complete semisynthetic diet mixed with an equal amount of bentonite by weight. The details of the experiments and the evidence that has led to the tentative conclusion that the hepatomas are the result, in part, of an induced choline deficiency are to be reported in detail elsewhere.[1, 2] Bentonite is a cation exchange silicate and apparently removes choline from the contents of the digestive tract so that it cannot be absorbed into the body, thus resulting in a deficiency.

Mice can adapt themselves to eating a diet containing half its weight of such inert material as Ruffex or barium sulphate with only a slight decrease in growth rate (FIGURE 1). Where bentonite makes up 25 per cent of the diet, growth is also nearly normal; but with 50 per cent bentonite in the diet, growth is almost completely suppressed (FIGURE 2). The mice continue to live, however, and grow very gradually for several months, but do not reach a normal size. Since the mice eat as much food on the 50/50 Bentonite diet as on the 50/50 Ruffex diet, failure to grow is not due to inanition alone.

Histological studies reveal that after the first three weeks, there is more or less fat in the livers (PLATE 1; 1), apparently depending on whether or not the mouse is gaining weight. This is in keeping with the general observation that, even on a diet free of lipotropic substances, the liver may not contain fat if the animal is not growing. The fat appears first as fine droplets, but soon many of the liver cells are distended with single large drops. Eventually, after three or four months, there is some fibrosis with progressive loss of architecture (PLATE 1; 2), although typical "hob-nail" cirrhosis has not been seen. There is also the accumulation of masses containing a brownish pigment, apparently ceroid (PLATE 1; 3).

Hepatomas have been found in all but one of twelve mice that in three experiments have been kept over 200 days on the diet. These are typical benign hepatomas of mice, much like those produced by repeated CCl_4 treatment or occurring spontaneously in C_3H mice.

The fatty livers, fibrosis, ceroid, and hepatomas present a picture much like that we have previously reported in mice maintained for several months on a diet low in protein and deficient in choline.[3] The symptoms develop more gradually and the mice maintain a more thrifty appearance. On a choline-deficient diet, at sporadic intervals, individual mice suddenly become anemic and die, so that only a few have been kept on it long enough to develop hepatomas. It seems possible that, in these sudden deaths, toxic materials produced by the intestinal flora may be involved. If so, they may be removed by the bentonite, which might account for the more thrifty condition of animals on the 50/50 bentonite diet.

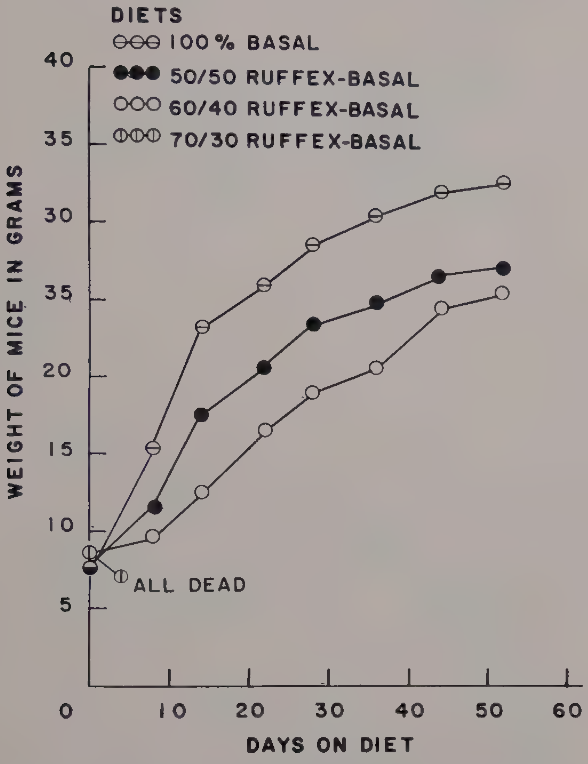

FIGURE 1

We have found that added choline will prevent the development of the fatty livers on the 50/50 bentonite diet, but that normal growth is only partially restored (FIGURE 2). When the amount of casein is doubled at the expense of corn starch, growth is also partially restored and the livers do not become fatty (FIGURE 3). This would be true of choline deficiency in both cases. When 1 per cent dl-methionine is added to the 50/50 bentonite-basal diet, however, the mice develop fatty livers and fail to grow (FIGURE 4). As a tentative explanation of this anomalous result, we suggest that the bentonite removes dibasic amino acids, and that the added methionine precipitates an incipient amino acid imbalance. These mice do not survive for long periods, the longest about 10 weeks, and with little gain in weight. It is only by nursing them along in a warm chamber at 31°C. that they can be maintained for any length of time at all. They have also developed a scaly tip to the tail, somewhat like that described for rats deficient in essential amino acids, which we have not as yet been able to cure.

It seems, then, that the deficiency is not a simple choline deficiency but may involve other essential basic materials removed from the intestinal contents by bentonite. It would be expected that some of the larger basic molecules would be taken up and held more firmly than inorganic cations, for with

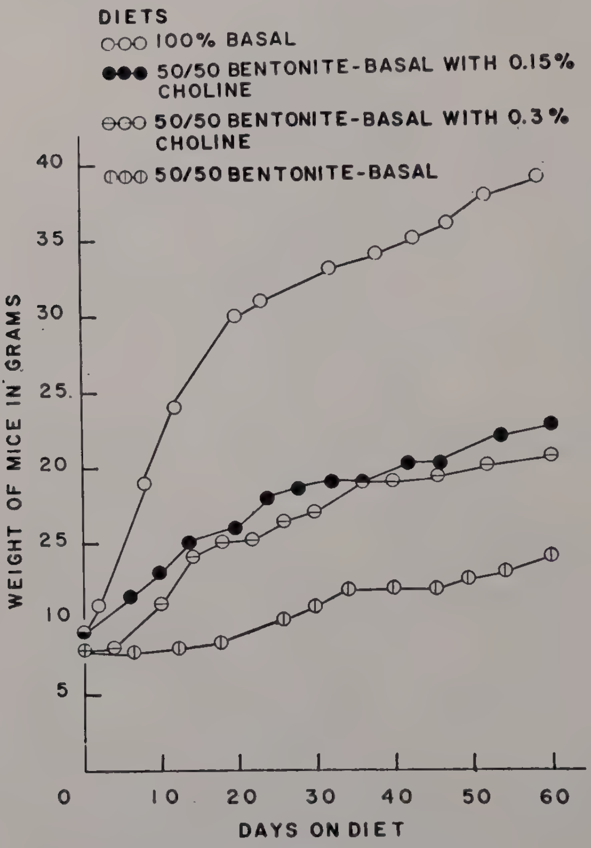

FIGURE 2

them, in addition to the Coulomb forces, Van der Waals forces come into play, and bases so held are only with difficulty eluted.[4] It would be difficult to predict what would be taken up under such complicated conditions as occur in the passage of the digestive tract. Experiments designed to test various possibilities are under way.

Except for the appearance of fat, there are no striking histological changes for the first four weeks. By the 60th day, there is a pronounced increase in the size of the nuclei, particularly in the centrolobular area (PLATE 1; 1–2). The cells are very much enlarged and the cytoplasm homogeneously finely granular and eosinophilic. The basophilia, usually conspicuous in the cytoplasm in this region, is absent and there are no glycogen vacuoles. Particularly in the animals with methionine added to the diet, there are no fat vacuoles because any mouse that has survived this long is no longer growing at all. Many of the enlarged nuclei contain conspicuous eosinophilic inclusions (PLATE 1; 4). Similar large cells with large nuclei and nuclear inclusions occur in the livers of

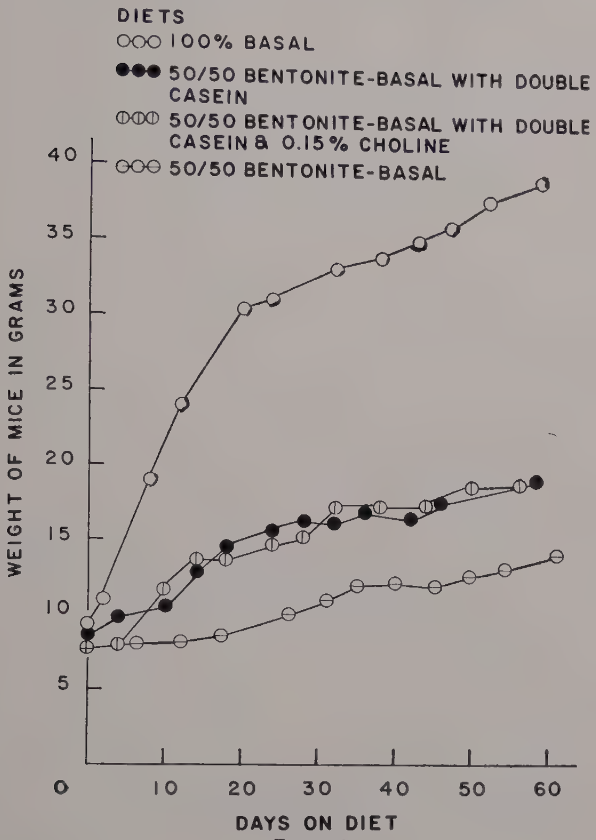

FIGURE 3

mice when they have been fed a diet containing a carcinogenic azo dye for a few weeks.[5]

The livers in which these enlarged cells appear are, in fact, very small. Apparently, there has been a gradual loss of cells with attempted replacement by cellular enlargement rather than by cell division. There are occasional mitotic figures throughout the series, but they are never abundant. When mice which have been 60 days on the 50/50 bentonite-basal diet and which have these enlarged liver cells are placed on a normal diet, rapid growth resumes and a spectacular wave of mitosis occurs in the liver which involves the large cells as well as the small ones (PLATE 2; 3). The large nuclei are obviously polyploid, for they have very wide-angled spindles and very large numbers of chromosomes. The metaphase illustrated in PLATE 2; 3 has a few chromosomes that have failed to reach the equatorial plate. From their size, the numbers on the plate must be great. The normal number for the mouse is 40.

Many of the figures are abnormal, with chromatin bridges persisting at anaphase and telephase (PLATE 2; 4). We have described abnormal figures of

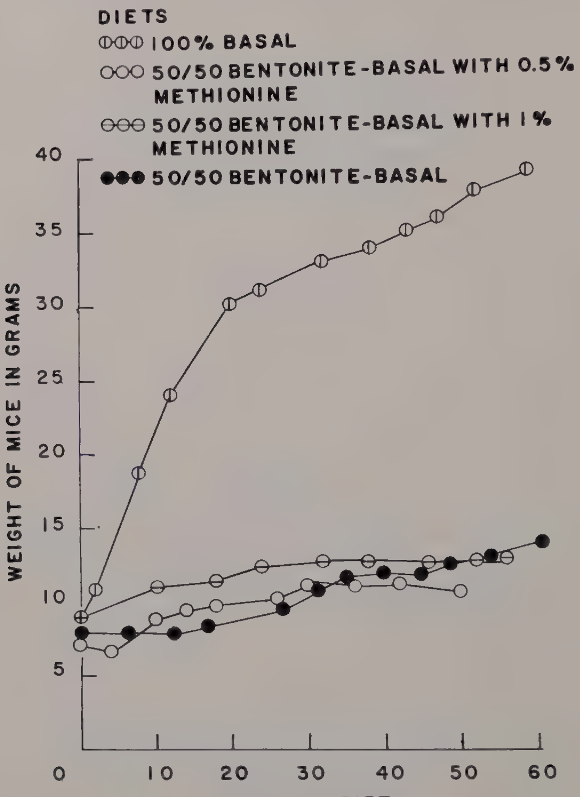

FIGURE 4

this sort in other cases where mitosis occurs in the mouse liver.[6] They occur also when the liver, regenerating after partial hepatectomy, is irradiated. Whether they result in viable cells with abnormal chromosome complements and thereby play a role in carcinogenesis as Brues suggests,[7] is an interesting speculation, and is worthy of further investigation particularly in light of the observation of Hauschka and Levan[8] on the occurrence of abnormal chromosome numbers in ascites tumors of mice.

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PLATE 1

1. Fatty liver of mouse on 50/50 bentonite-basal diet with 1 per cent methionine for 49 days. Ocular 12.5 and objective 20×.

2. Loss of lobular architecture after 280 days on the 50/50 bentonite-basal diet. Ocular 7.5 and objective 20×. Fat vacuoles and some fibrosis. Masses with smaller vacuoles in the center contain brownish pigment, apparently ceroid.

3. One of the masses apparently containing ceroid from the liver in FIGURE 2. Ocular 7.5 and objective 120×.

4. Nuclear inclusions in enlarged liver cells of mouse on 50/50 bentonite-basal with 1 per cent methionine for 56 days. Ocular 12.5 and objective 20×.

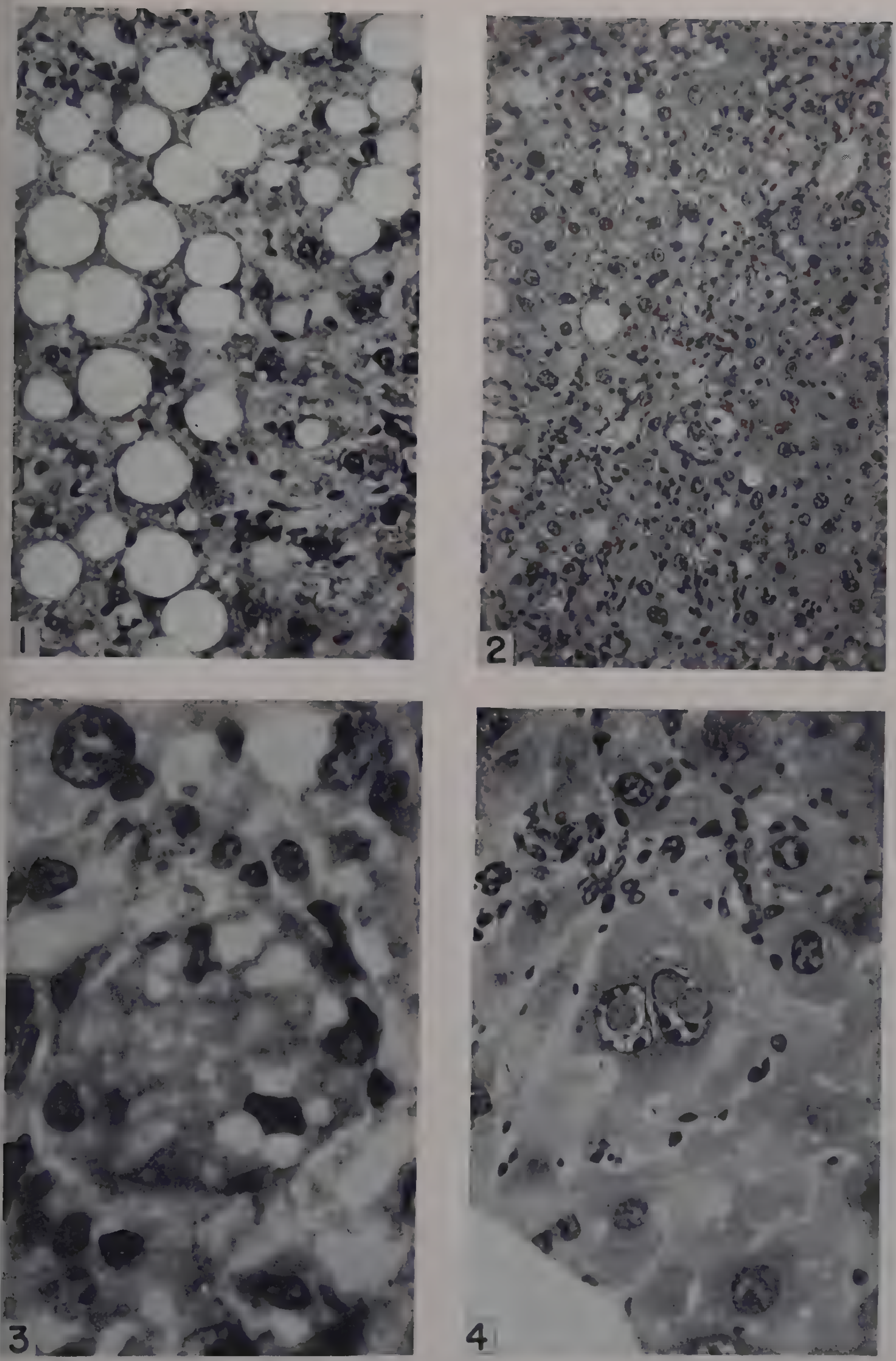

PLATE 1

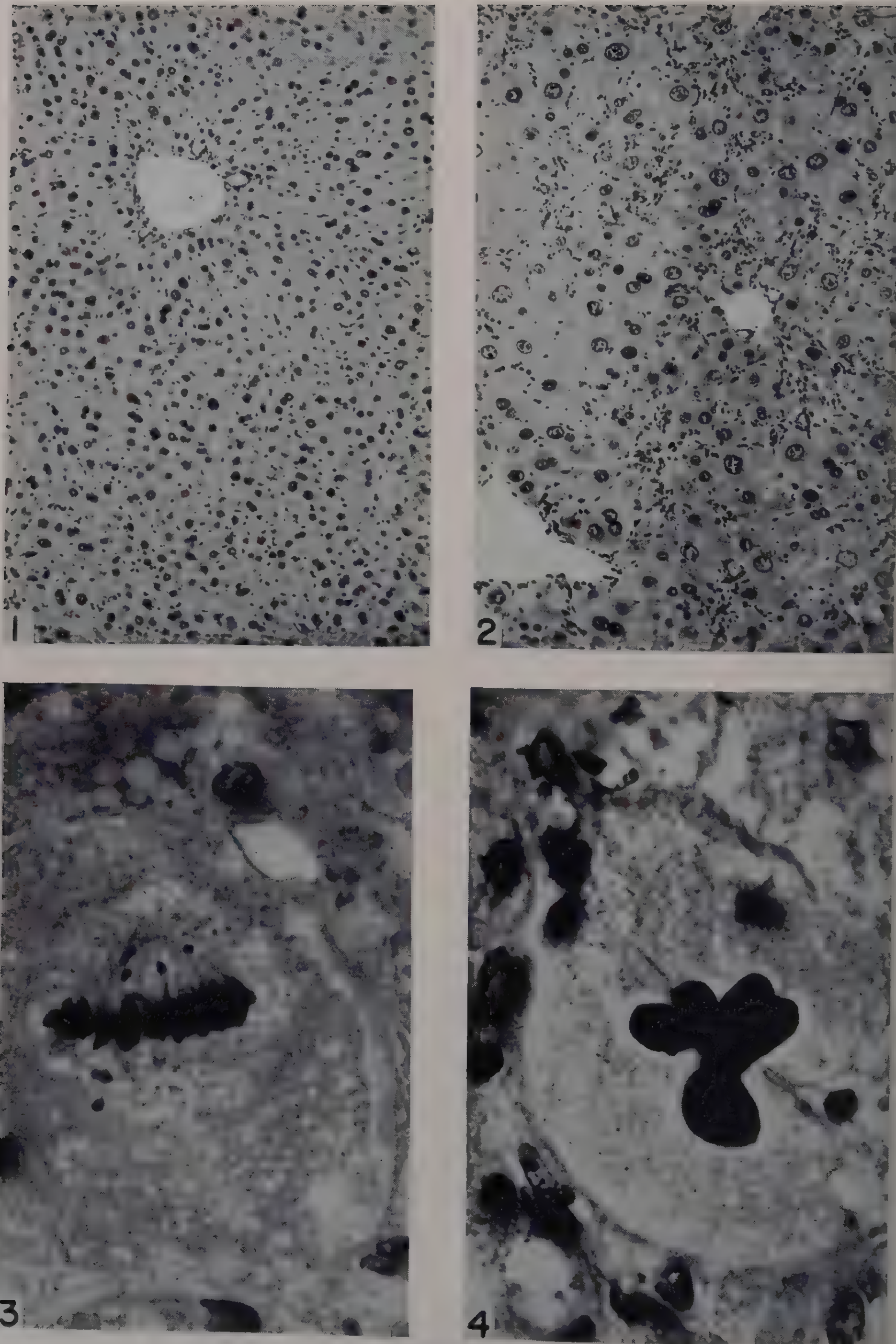

PLATE 2

PLATE 2

1. Liver of mouse after 60 days on 50/50 bentonite-basal diet with double casein. 12.5 ocular and objective 20×. Normal in appearance.

2. Liver of mouse after 56 days on 50/50 bentonite-basal with 1 per cent methionine. 12.5 ocular and objective 20×. Nuclei very much enlarged.

3. Metaphase in enlarged liver cell of mouse after 56 days on 50/50 bentonite-basal with 1 per cent methionine 7.5 ocular and objective 120×. Wide angled spindle with scattered chromosomes.

4. Abnormal telophase in enlarged liver cell of same mouse as FIGURE 3. Telophase nuclei have failed to separate and are interfering with cleavage furrow.

THE EFFECT OF AUREOMYCIN ADMINISTRATION UPON THE BLOOD LIPIDS OF DOGS WITH LIGATED PANCREATIC DUCTS*

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Introduction

Dogs deprived of the external secretion of the pancreas but receiving an adequate insulin supply develop a fatty liver,[1-5] exhibit great reduction in blood lipid levels,[6, 7] and manifest a marked elevation in serum alkaline phosphatase activity.[8] These changes are characteristic of both the insulin-treated depancreatized dog and the dog with completely ligated pancreatic ducts. Effective lipotropic substances[9-15] restore the liver fat content to normal, raise the blood lipids, and decrease the serum alkaline phosphatase activity.

Heretofore, the only identified substances reported as having a preventive or therapeutic effect upon such dogs have been choline, its precursors or proteolytic enzymes, that liberate the methionine present in ingested protein.[9, 11-18] In this respect, an anomaly was recently resolved by Haanes and György[16] who demonstrated that an effective lipotropic fraction of pancreas that previously had been considered as having no proteolytic activity was actually a compound of trypsin and inhibitor that exhibited enzymatic activity after incubation with duodenal juice or intestinal mucosa.

Despite the fact that dogs deprived of the pancreatic exocrine secretions digest and absorb from 30 to 70 per cent of the protein in the diet, all attempts to prevent fatty infiltration of the liver by feeding extra protein have resulted in failure even though the meat or protein content of the diet were doubled.[13] Ralli and Rubin,[19] however, found that the feeding of a powder prepared from lean meat that had been extracted with water produced a fatty liver much more slowly and to a lesser degree than the feeding of the powder plus the aqueous extract or fresh meat alone. These findings raise the possibility that an excess of compounds requiring methylation may be present in the intestinal tract when the digestion of protein is incomplete, thereby increasing the demand for choline or methionine. Moreover, in the absence of pancreatic enzymes, the intestinal bacteria themselves may destroy or utilize increasing amounts of choline or methionine. Both processes would leave less choline available for lipotropic purposes.

In order to evaluate the role that the intestinal bacteria of pancreatic duct-ligated dogs play in the relative choline deficiency of these animals, an attempt was made to alter the flora by the oral administration of antibiotics. The success of György and his associates[20-22] in using antibiotics to inhibit the formation of hepatic dietary necrosis or the production of fatty infiltration of the liver by dietary means led us to investigate the effect of oral aureomycin upon the lipid metabolism of dogs with ligated pancreatic ducts. Preliminary

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* Aided by Grant G-3275 from National Institutes of Health, U. S. Public Health Service.
† This department is supported in part by the Michael Reese Research Foundation.

results indicate that supplementation of the diet with aureomycin has a lipotropic effect.

Procedure

The pancreatic ducts of adult dogs were ligated, the pancreatic tissue stripped from the duodenum, and omentum interposed and sewn in place, according to the technique of Montgomery *et al.*[5] The animals were fed twice daily a meal consisting of 300 gm. of horse meat, 50 gm. sucrose and 2 gm. of salt mixture. Twice a week this was supplemented with 5 cc. of a rice bran concentrate and 3 cc. of cod liver oil. The studies reported were performed on dogs whose pancreatic ducts had been ligated one to two years prior to the administration of aureomycin.

During the period of aureomycin* administration, antibiotic capsules were given to 3 dogs in doses of 0.5 gm. twice daily (with each meal) for the first 3 weeks, and then 0.25 gm. 3 times daily (with each meal and once at night).

Blood for analyses was obtained in the postabsorptive state. Serum cholesterol was determined by the Sperry and Webb modification[23] of the Schoenheimer-Sperry method.[24] Lipid phosphorus was estimated in an alcohol-ether extract of serum by the method of Zilversmit and Davis.[25] Serum alkaline phosphatase activity was measured by the method of Kaplan and Narahara,[26] in which 1 unit is equivalent to approximately 0.8 Bodansky units. Ergothioneine in urine was estimated by the method of Sullivan and Hess.[27]

Results

The oral administration of aureomycin to three pancreatic duct-ligated dogs was accompanied by a rise in serum total cholesterol and ester cholesterol levels that were paralleled by an increase in phospholipids, while the alkaline phosphatase activity dropped. These alterations reached a maximum in 3 or 4 weeks, but then regressed and reached a plateau. Montgomery *et al.*[5] have shown that the pancreatic duct-ligated animal usually has a fatty infiltration of the liver within 20 weeks of the operation, a finding that previously had been reported for the completely depancreatized dog maintained with insulin.[2] In the present study, the animals were duct-ligated from 44 to 100 weeks prior to the antibiotic administration and exhibited the greatly lowered serum concentration of cholesterol and phospholipids and the elevated alkaline phosphatase typical of this condition. The results obtained in each of the three dogs were as follows:

Dog No. 2155. This animal had pancreatic duct ligation performed one year prior to this experiment. Within one week of aureomycin administration, a rise in the serum lipids and a fall in alkaline phosphatase were evident (FIGURE 1). These changes reached a maximum in three weeks, at which time the total cholesterol had risen from 38 to 159 mgm. per cent, ester cholesterol from 26 to 111 mgm. per cent and lipid phosphorus from 3.7 to 9.6 mgm. per cent. During this period, the alkaline phosphatase activity had fallen from 65 to 16.7 units. Despite continued aureomycin administration, however, a

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* We are indebted to Doctor S. M. Hardy, Lederle Laboratories Division, American Cyanamid Co., for the generous supply of aureomycin.

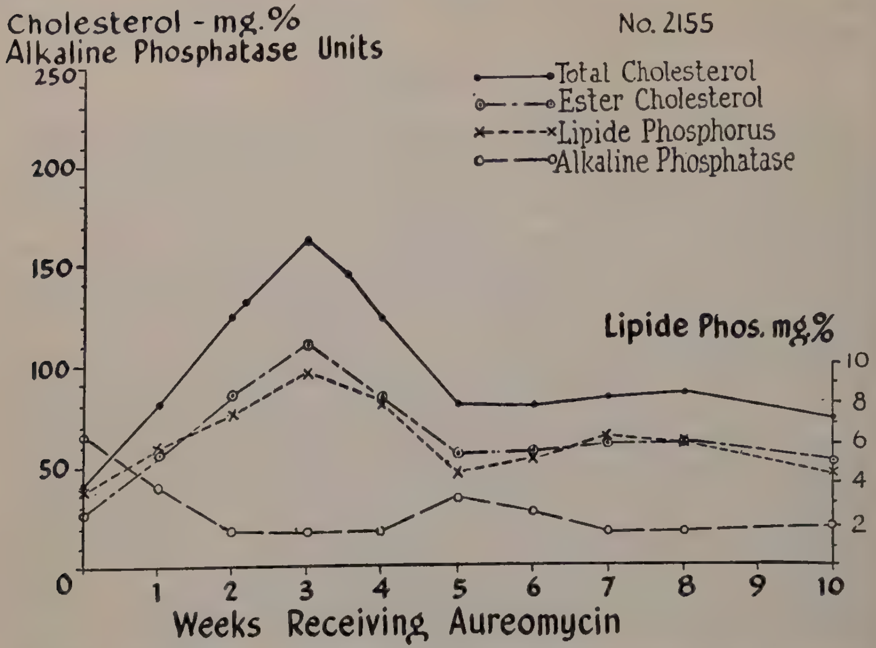

Figure 1. Effect of aureomycin *per os* upon the serum lipids and alkaline phosphatase activity of pancreatic duct-ligated dog no. 2155.

regression took place and the concentrations of serum lipids leveled off at approximately 50 per cent of the peak values. These were still well above the control concentrations. The serum alkaline phosphatase activity rose somewhat during this period but did not approach the initial value.

Dog No. 2661. The pancreatic ducts of this dog were ligated 44 weeks prior to the antibiotic experiment. Like the preceding animal, this dog responded to aureomycin administration with a rise in total cholesterol, most of which was due to an increase in the ester portion, a rise in lipid phosphorus and a fall in serum alkaline phosphatase (figure 2). The maximum changes were reached in four weeks but were not as marked as in the previous animal, corresponding more closely to the plateau values. These alterations, however, were sustained for an additional 10 weeks, at which time aureomycin was withdrawn from the diet. There was an immediate fall in serum lipid concentration and a rise in alkaline phosphatase activity, approximating the control values in three weeks.

Dog No. 1921. The pancreatic ducts of this dog were ligated two years previous to this experiment. He showed the customary low blood lipids and high serum alkaline phosphatase activity (figure 3). Three weeks after the administration of aureomycin, the serum cholesterol and lipid phosphorus returned to normal and the alkaline phosphatase activity dropped considerably. As in dog 2155, these values were not maintained, and the serum lipids reached a plateau intermediate between the control and peak levels. At this time, the animal was given daily 6 mcg. of vitamin B_{12} subcutaneously and 5 mgm. of

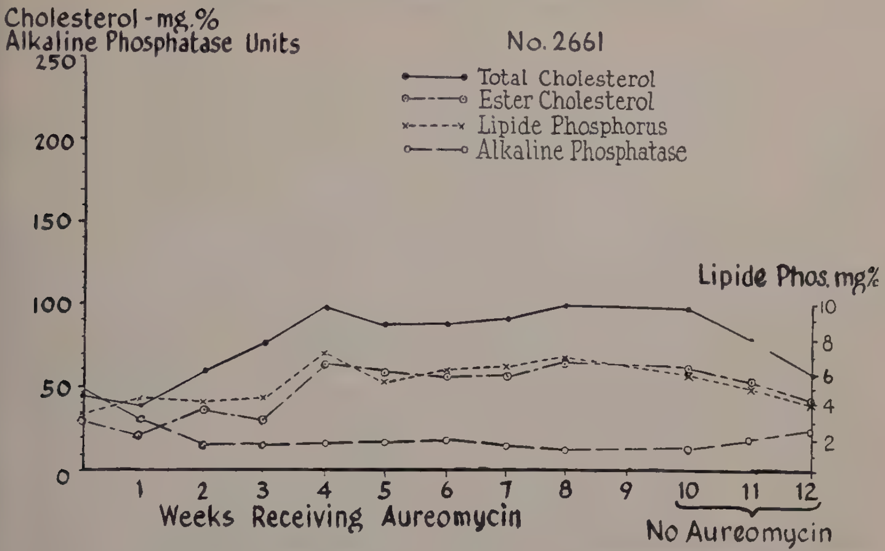

FIGURE 2. Effect of aureomycin *per os* upon the serum lipids and alkaline phosphatase activity of pancreatic duct-ligated dog no. 2661.

folic acid orally in addition to the aureomycin. The serum cholesterol and lipid phosphorus promptly rose to peak levels and were maintained at this point for three weeks. Alkaline phosphatase activity also fell to its lowest level. Upon withdrawal of the aureomycin from the diet while continuing the above vitamin administration, the serum lipids fell and the alkaline phosphatase activity rose. These results indicate that the lipotropic action of aureomycin exceeds that of the vitamin B_{12} and folic acid. It should also be pointed out that, one year previously, no effect on serum lipids or alkaline phosphatase activity was obtained in dog 1921 or in one other animal by the oral administration of 25 mcg. of vitamin B_{12} plus 5 mgm. of folic acid, nor by the subcutaneous administration of 100 mcg. of vitamin B_{12} alone, or 6 mcg. of vitamin B_{12} plus 4.5 mgm. of folic acid. No antibiotics were given at that time.

In searching for the mechanism by which aureomycin was exerting its lipotropic effect, consideration was given to the possibility that, through its antibacterial action, less methyl acceptors may be formed. Although little is known concerning the metabolism of ergothioneine, the betaine derivative of thiolhistidine, it appeared likely that a precursor of ergothioneine may undergo methylation in the body. Accordingly, the urinary excretion of ergothioneine was estimated. As seen in TABLE 1, aureomycin did not decrease the weekly excretion of ergothioneine. The possibility of other methyl acceptors being involved in this condition cannot be eliminated.

In view of the reports by Huerga and Popper[28, 29] that the major portion of a test meal of choline is converted to trimethylamine and its oxide by intestinal bacteria and that this conversion can be temporarily inhibited by pretreatment

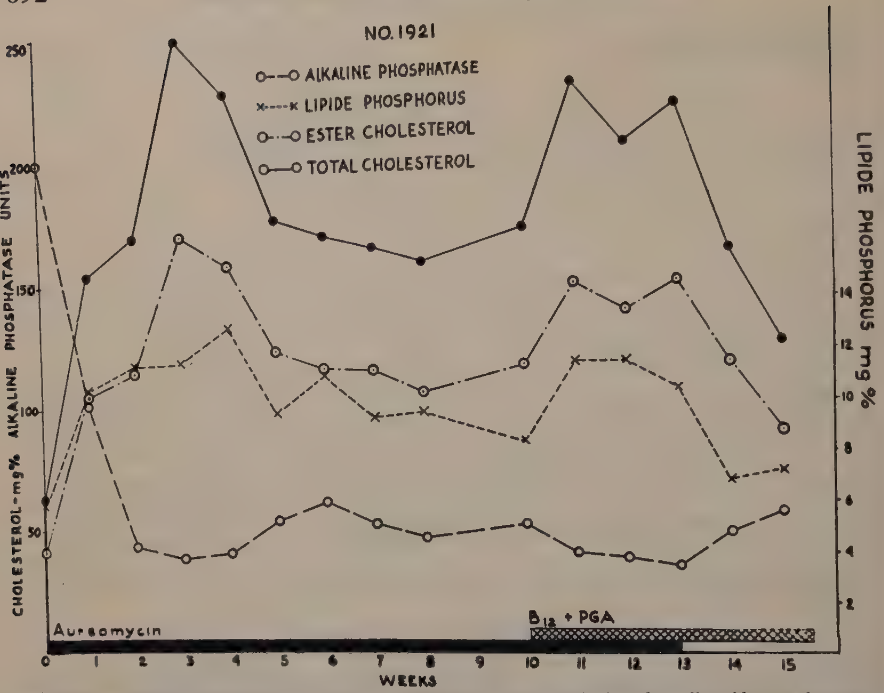

FIGURE 3. Effect of aureomycin alone and in conjunction with vitamin B_{12} plus folic acid upon the serum lipids and alkaline phosphatase activity of pancreatic duct-ligated dog no. 1921.

TABLE 1

THE EFFECT OF AUREOMYCIN INGESTION UPON THE EXCRETION OF ERGOTHIONEINE BY DOGS WITH LIGATED PANCREATIC DUCTS

Weeks receiving aureomycin*	Weekly excretion of ergothioneine (mg.)		
	Dog 2155	Dog 2661	Dog 1921
none	862	810	725
1	1254	598	978
2	900	1078	888
3	1652	1444	655
5	1011	1373	
6			922

* 1.0 g. daily for the first 3 weeks and 0.75 g. daily for the remainder.

with antibiotics, some measurements* of total trimethylamine excretion were made in some of our treated animals. No consistent depression of choline destruction was found during prolonged aureomycin therapy. If less choline were being converted to trimethylamine or its oxide, the action was temporary and of insufficient magnitude to explain the lipotropic effect of the antibiotic.

Discussion

These limited data indicate that aureomycin does have a lipotropic action when given *per os* to dogs with ligated pancreatic ducts, as shown by a marked rise in serum cholesterol and phospholipid levels and a dramatic fall in serum alkaline phosphatase activity. Conclusive demonstration of a lipotropic effect, however, depends upon liver biopsy studies. Such experiments are under way.

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* We are indebted to Dr. J. de la Huerga of Hektoen Institute, Chicago, Illinois, for these analyses.

The inability of aureomycin to maintain a normal level of serum cholesterol and phospholipids, as do lipotropic substances such as choline or methionine, may be explained by partial bacterial adaptation to the antibiotic. In view, however, of recent reports[30, 31] that the administration of aureomycin or terramycin may cause a fine, fatty infiltration of the liver, this latter possibility has to be taken into consideration. Nevertheless, it appears from our limited data* that fatty infiltration of the liver induced in the dog by the antibiotic *per se* is minimal, if present at all, but more animals are needed to settle the question. This is in agreement with the finding of Lepper *et al.*[32] that a very large oral dose of antibiotic is required to produce fatty liver changes in mice.

György and his associates were the first to demonstrate that aureomycin and other antibiotics exert a lipotropic action when fed to rats receiving a low-protein, low-choline, high-fat diet[22] and that the same antibiotics delay the onset of liver necrosis when rats are fed a necrogenic diet.[20, 21] The recent reports of György[22] and of Luckey[33] that rats raised in a germ-free atmosphere and fed the necrogenic diet show no signs of liver necrosis is convincing evidence that the intestinal flora play a role in the development of this condition. It also appears likely that the lipotropic action of aureomycin, whether obtained in rats suffering from a choline deficiency or in dogs with a deficiency of pancreatic exocrine secretions, is associated with its antimicrobial action. This lipotropic action may represent the summation of several processes, unknown at present, whereby choline or methionine may be spared to the body. Alterations in intestinal flora induced by antibiotics may result in the decreased formation of substances combining with methionine or in the decreased utilization of choline and methionine by the bacteria themselves.

Schaefer *et al.*[34] and Burns and McKibben[35] have reported a lipotropic effect for vitamin B_{12} when given to puppies raised on a choline-deficient diet. In our hands, the administration of large doses of vitamin B_{12} plus folic acid by both oral and subcutaneous routes failed to raise the low serum levels of cholesterol and lipid phosphorus in pancreatic duct-ligated dogs. When given with aureomycin, however, at a time when the lipotropic effect of the latter had declined and leveled off, the administration of the above vitamins produced a sharp, sustained lipotropic action in one dog. At a later date, two other dogs showed no response to these vitamins even though they were administered under the same conditions. This suggests that the degree of choline deficiency or the bacterial flora of the intestine may be factors influencing the lipotropic effect of vitamin B_{12} and folic acid.

Summary

(1) The oral administration of aureomycin to dogs with ligated pancreatic ducts has a lipotropic effect as manifested by a marked rise in the existing low concentrations of serum cholesterol and lipid phosphorus to values approaching normal. The elevated serum alkaline phosphatase activity falls sharply.

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* A normal dog was given by mouth 1.0 gm. aureomycin daily for 3 weeks and 0.75 gm. daily for an additional 2 weeks before it was sacrificed. Total liver lipids were at the upper limit of normal, namely 4.9 per cent.

(2) These lipid and enzyme changes reach their maximum in three or four weeks and then recede and level off at some intermediate value.

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SURVEY OF THE WORLD SITUATION ON KWASHIORKOR

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A world survey is of value in this scientific monograph because the study of the ecology and epidemiology of kwashiorkor* may provide useful clues to the etiology and pathogenesis of cirrhosis and primary carcinoma of the human liver.

The term "kwashiorkor" now has, for better or for worse, the sanction of the Joint F. A. O./W. H. O. Expert Committee on Nutrition, which has quoted and presumably endorsed the view expressed by Brock and Autret that "a wide and general appraisal of its nature and relationships suggests that it is the most serious and widespread nutritional disorder known to medical and nutritional science." In the African continent, the term has been accepted almost unanimously as replacing "malignant malnutrition," "infantile pellagra," "diboba," "mbuaki," and other local names.

No attempt will be made in this communication to trace the history and details of this syndrome and its bibliography except in so far as they are relevant to the title of this monograph. Brock and Autret[5] have done this and have made acknowledgment to the pioneers in this field in Africa. They defined kwashiorkor as seen in the African continent in the following terms: 'It is recognized that the syndrome cannot at present be accurately defined and that it merges into other nutritional syndromes, such as marasmus and Mehlnährschaden, but it is felt that its prevalence and importance justify a tentative definition as follows:

"A nutritional syndrome (or syndromes) found among indigenous Africans in which characteristically there occurs:

"(a) retarded growth in the late breast-feeding, weaning, and post-weaning ages, with
"(b) alterations in skin and hair pigmentation,
"(c) oedema,
"(d) fatty infiltration, cellular necrosis, or fibrosis of the liver,
"(e) a heavy mortality in the absence of proper dietary treatment, and
"(f) the frequent association of a variety of dermatoses.

"Some combination of the above features appears to be fundamental; other clinical features are so frequent as to be dominant if not fundamental."

The African aspects of kwashiorkor were brought up to date by the Second Conference on Nutrition called by the Commission for Technical Cooperation in Africa South of the Sahara (C. C. T. A.) in November 1952 at Fajara, Gambia, West Africa.[7] The Joint F. A. O./W. H. O. Expert Committee on Nutrition[19] held its third session at Fajara immediately after the C. C. T. A. Conference. The members of the Committee had the advantage of hearing the full discussion at the C. C. T. A. Conference and subsequently considered kwashiorkor in relation to the problems of nutrition in mother and child throughout the world.

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* As far as concerns the relevance of kwashiorkor to the title of the Conference on which this monograph is based, the syndrome has been referred to in the Transactions of several of the Conferences on Liver Injury called by the Josiah Macy, Jr., Foundation,[21] and particularly in the Report of the Ninth Conference. Section V on liver disease in Jamaican children in the report of the Tenth Conference (1951) is also relevant to the following discussion.

In the light of these two meetings, the definition and description given by Brock and Autret can be modified or expanded in certain respects. The curative effect of reconstituted skim-milk powder has been amply confirmed and this might be listed as a fundamental and often critical diagnostic feature. As predicted by Brock and Autret, vegetable protein has proved to be capable of substitution to a limited extent for milk protein.[10] We can conclude, therefore, that the deficient factor or factors are among the constituents of dried skimmed milk. Whether the deficiency pattern is (1) a simple disproportion between total protein and calories, or (2) an amino-acid imbalance, or (3) a deficiency of accessory food factors yet to be identified, remains an open question. The Joint F. A. O./W. H. O. Expert Committee on Nutrition has very properly left this matter open in its tentative definition of "protein malnutrition."

There is no further confirmation of the claim by Auffret and Tanguy[1] that the milk of African mothers is deficient in methionine. Bigwood[3] has pressed the importance of cystine deficiency. Trowell, Davies, and Dean[28] have brought their description of kwashiorkor in Uganda up to date. Davies[8] has given an account of the pathology of the syndrome as seen in Uganda. He believes that certain features are pathognomonic, especially the pancreatic lesion and the "stellate" fibrosis of the liver. He holds that atrophy of the acinar cells of the pancreas with marked decline in the extrinsic secretions of the gland is a fundamental feature of the syndrome and perhaps the essential and earliest lesion. If Davies' observations and interpretations are correct, and if they apply to kwashiorkor as seen in other regions then the pathogenesis of the syndrome can be stated in some such terms as the following: Under the influence of protein malnutrition which is acutely precipitated by weaning, the pancreas and other organs of exocrine secretion are first affected. This leads to decline in enzymatic and other efficiency of the gastrointestinal tract and creates a vicious circle of conditioned and dietary protein deficiency, which, in turn, affects the liver and every tissue in the body.

Turning from Africa to other parts of the world the incidence of kwashiorkor was reviewed by Meiklejohn and Passmore[22] and has recently been brought up to date.[28, 19b] A survey of kwashiorkor in Central America has been made by Autret and Behar and will be reported. There is a general impression that kwashiorkor is a syndrome apparently basically the same as "culebrilla," "syndrome pluricarencial," "syndrome hipoproteinico" and related syndromes of Central and South America, and the "fatty liver disease" of Jamaica. Apart from the pigmentary disturbances of the skin which are usually present, it is probably basically the same as the "Mehlnährschaden" of Germany and Austria in the early 1900's, and the "dystrophie de farineuse" and "distrofia de farinacei" still recognized in the unpigmented or lightly pigmented children of southern Europe. The report of the third session of the Joint Committee (November 1952) contains a comprehensive list of titles which the Committee believes to signify syndromes of the same nature as kwashiorkor and covered by the general term "protein malnutrition" as defined in the report: ". . . a state of ill-health occurring where diets are habitually poor in good quality protein, while they are more nearly adequate in calories. It includes deficiency of protein foods, imbalance of amino acids, and deficiency of accessory food factors associated with protein metabolism. It is most easily recognized when calorie intakes have been relatively high from the use of starchy foods."

Because no one name commands general acceptance to cover the "protein malnutrition" syndromes of the American tropical belt and because the Joint F. A. O./W. H. O. Expert Committee on Nutrition has been inclined to include them under the heading "kwashiorkor," I have compromised in this communication by grouping them for convenience under the term "American kwashiorkor." I could not use the plain term "kwashiorkor" because, on account of the apparent difference in sequelae, I have asked the question whether kwashiorkor and "American kwashiorkor" are the same disease? In spite of difficulties which will be discussed, I believe the answer to be in the affirmative.

A brief summary will be given of present knowledge in regard to the liver lesion or lesions associated with kwashiorkor. This falls under two headings: (1) the pathology of the liver in kwashiorkor in childhood; and (2) the liver lesions of adults in the areas of prevalence of kwashiorkor and protein malnutrition and the possible relationships between these adult lesions and those of the children. Brock and Autret reported that the liver pathology of African kwashiorkor consisted of some combination of fatty infiltration, necrosis, and fibrosis. These three features showed various combinations, and there is much experimental evidence that all three may occur separately and independently as manifestations of dietary deficiency. The experimental evidence for the separate origins of fatty infiltration and necrosis are reviewed elsewhere in this monograph.† There can be no doubt that the processes described under these two terms are quite distinct, but it should be emphasized that even the "dissolution of fatty cysts" described by Hartroft in his lipohepatosis is a form of death of cells. It was in this wide sense that the liver lesion of kwashiorkor was described by Brock and Autret as "fatty infiltration, cellular necrosis, or fibrosis." It was intended to leave the pathogenesis an open question. The available evidence certainly suggests close analogies between the liver lesion of kwashiorkor and experimental fatty liver cirrhosis, but it would be premature to press this comparison too far. This subject is discussed further in the section on definitions (Appendix, p. 709). Workers on kwashiorkor in Africa have inclined strongly to the concept of the independence of fatty infiltration and of fibrosis. Both Gillman[13] and Davies[8] have recorded their views on this subject. There seems to be no reason why the fatty infiltration of kwashiorkor should not be permanently reversible on provision of a proper diet, and the problem of continuing fibrosis is discussed in a later section of this communication.

Brock and Autret in their report stated that "it can be accepted as reasonably probable, from their world distribution, that cirrhosis and primary carcinoma of the liver on the one hand, and kwashiorkor on the other hand, are aetiologically related. The evidence strongly suggests that both are due to dietary deficiency, particularly deficiency of protein." The evidence for this statement is worth critical examination, since it is based on some tentative assumptions.

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† See page 623.

The areas of the world in which kwashiorkor is prevalent are very similar to but not identical with the areas in which cirrhosis and primary carcinoma of the adult human liver have a high incidence. The areas of correspondence are readily explainable on the attractive hypothesis that the gross hepatic damage of infantile kwashiorkor is the starting point of a fibrosis which proceeds insidiously throughout adolescence and presents itself clinically in the third and fourth decades of life as a "nutritional cirrhosis" which is often complicated by primary carcinoma of the liver. This hypothesis is at present only provisional. Careful examination of maps of the world areas of noncorrespondence between infantile kwashiorkor on the one hand and "nutritional cirrhosis" and primary carcinoma of the liver in adults on the other may yield important clues as to the reliability of the assumption.

What is needed at present is a series of accurate maps of the world reflecting the areas of incidence of (1) kwashiorkor or related protein malnutrition syndromes, (2) prevalence of primary carcinoma of the liver, and (3) prevalence of cirrhosis of the liver. The first map has been constructed from the literature[7, 22, 28] but will need to be filled in as reports accumulate (FIGURE 1). A version of the second map is contained in Berman[2] (FIGURE 2).

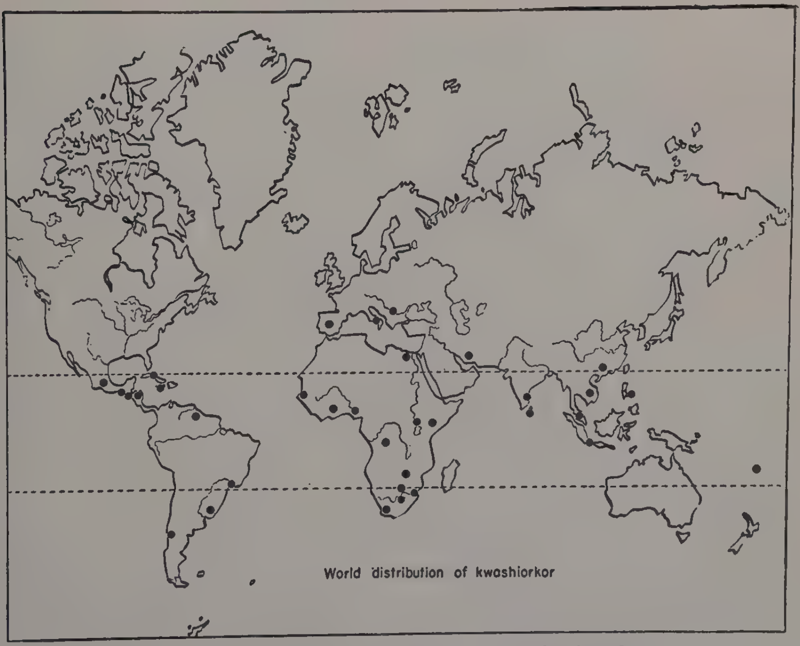

FIGURE 1. World distribution of kwashiorkor and related syndromes.

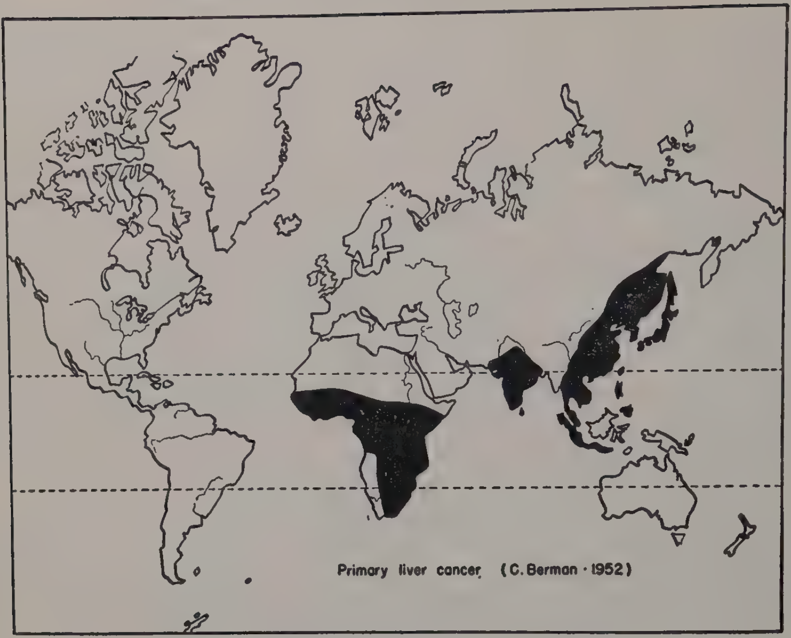

FIGURE 2. World prevalence of primary carcinoma of the liver.

Without detracting from the value of Berman's work, this map can be criticized both artistically and scientifically in its "black and white" character. The figures given in FIGURE 3, prepared by myself, show that the difference in incidence of primary liver carcinoma could be better represented by a gradual merging from black through grey to white as we pass from the tropics through the pigmented residents of temperate climates to the European races. Berman's map can also be criticized on the score that he produces no evidence that the prevalence of primary carcinoma admitted to exist in South China continues up to North China as represented on his map.

If these two maps are compared, there is considerable correspondence between the latter and the world map of kwashiorkor as far as Africa, India, China, and the Far East within the tropical belt are concerned, but a notable discrepancy with respect to the tropical belt of the American continents. Does this blank in the tropical portion of the map of incidence of primary liver cancer reflect actual conditions, or is it due to failure to record and report? Warvi[31] said, "Near and Far Eastern races have a greater incidence of liver cell carcinoma than those of Europe *or the Americas*." Berman's summary confirms this, and apparently there is no contrary evidence.

I have sought unsuccessfully in the literature for information on this point and have questioned and corresponded with those who might know. The problem ties in with the much more difficult problem of constructing a world map of the incidence of cirrhosis of the liver of all types. The best I have been able to do on this point is shown in FIGURE 4 which is admittedly incomplete.

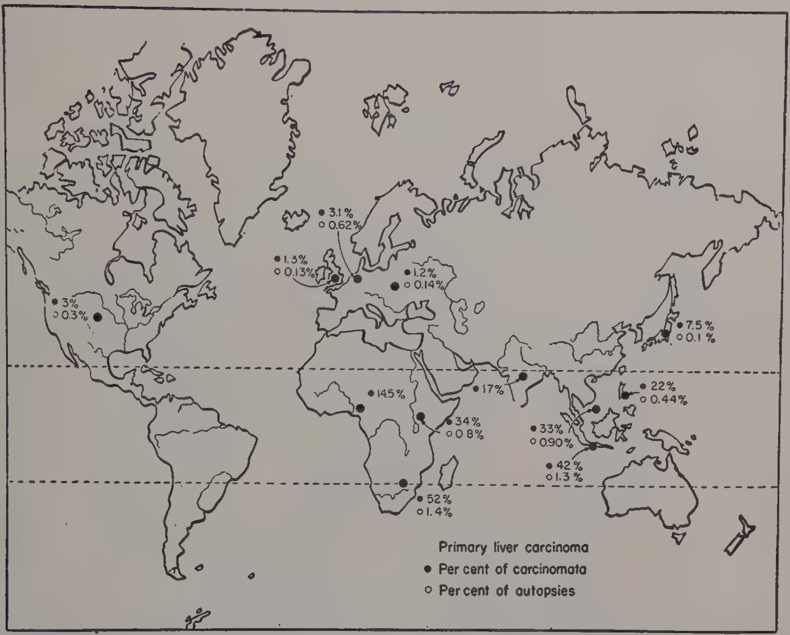

FIGURE 3. World incidence of primary carcinoma of liver expressed as percentages of all carcinomata and of autopsies.

This charting of geographical pathology is complicated by different concepts of cirrhosis (see definitions); but, in general, the figures represent macroscopic or naked-eye incidence. The open circles represent incidence rates of less than 5 per cent in general autopsies, and the black dots higher rates. It will be seen that the difference between the tropical and nontropical areas is considerable but not as gross as would be expected from the map of primary liver cancer. For interest in relation to later discussion, I have also charted the areas of prevalence of siderotic cirrhosis and of liver damage resulting from hepatotoxic alkaloids contaminating food.

The only data given for the American tropical belt are those derived from reports on the yellow-fever viscerotome service, a method whereby postmortem liver samples were obtained without doing an autopsy. The figure of 2 per cent for Colombia represents 404 cases with "signs of cirrhosis" out of 22,000 specimens.[12] That of 0.5 per cent for central and northern Brazil represents 55 livers with "advanced cirrhosis" out of 29,592 specimens.[9] It must be remembered that these were all microscopic specimens. If the data are based on the usual criteria, the incidence of cirrhosis is low in these two large countries of the central American belt. I have been able to find no publications on the cirrhosis rate for this area based on the usual autopsy incidence rate.

If microscopic criteria are taken for the diagnosis of cirrhosis, then the incidence becomes very much higher in some parts of the world. The Gillmans,[13]

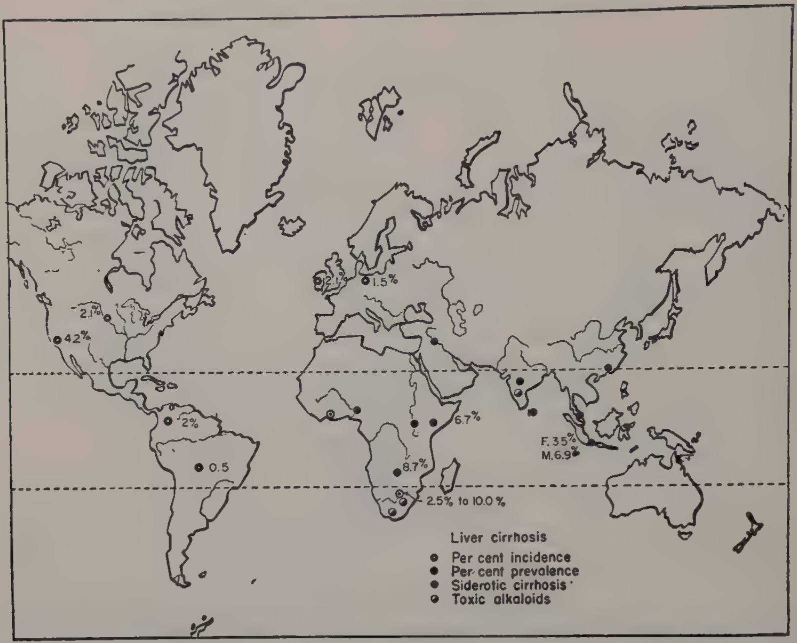

FIGURE 4. Liver cirrhosis expressed as a percentage of autopsies in areas of high and low incidence. Areas of liver damage associated with siderotic or iron-rich cirrhosis and with toxic alkaloids are shown.

for example, claim that among 261 adult African (Bantu) subjects dying "sudden and rapid accidental death" only 12.6 per cent of livers were histologically normal. Such a figure cannot be compared with figures from other parts of the world since histological criteria should be the same. That the Gillmans' criteria are different from those applied in the U. S. A. is suggested by their figure of 67.8 per cent normal livers among comparable Europeans in Johannesburg, while McCartney[20] claims that his microscopic cirrhosis rate is lower than his macroscopic rate of 2.5 per cent.

On the other hand, Bauer and Kerr, although they record only 55 cases of "advanced cirrhosis" in 29,593 specimens, nevertheless record that, in 1594 of those specimens in which lesions of *S. mansoni* were found, "periportal reaction" is always present and ranges from a slight hepatitis through various degrees of fibrosis with chronic inflammation to severe cirrhosis. And in 2298 further specimens showing malarial pigment, there were 23 per cent with "periportal reaction." Admitting that periportal reaction is not synonymous with cirrhosis, it seems that the figure of 55 cases of "advanced cirrhosis" in the whole series represents a very conservative estimate of the actual incidence of cirrhosis in Brazil.

The gap covering the American tropical belt in the world map of cirrhosis of the liver constructed from published information is very obvious, and again the question arises, as in the case of the map of primary carcinoma, as to whether this gap is real or only apparent? The opinion of some of those who have had experience is that it is only apparent. Until it is filled in, it cannot finally be concluded that "American kwashiorkor" is the same disease as kwashiorkor in Africa, for it appears, at present, not to have the same sequelae.

Interpretations

If there were any consequential relationship between kwashiorkor and primary carcinoma of the liver, that sequence would presumably be through the insidious development of cirrhosis of the liver as a sequel to kwashiorkor, because (1) the correspondence between primary carcinoma and cirrhosis of the liver is so close and (2) experimental evidence suggests that protein malnutrition in animals can result in progressive and permanent cirrhosis.

Two questions must be asked at this point: (1) What is the evidence that kwashiorkor in African can produce permanent cirrhosis of the liver? (2) What is the evidence that "American kwashiorkor" can produce cirrhosis of the liver? It will be seen in the later discussion that a final answer cannot be given to either of these two questions.

The next question to be asked is: What other factors besides protein malnutrition may enter into the production of cirrhosis of the liver either as basal causative factors or as contributory factors? In the later discussion, this aspect of the problem will be treated under the following headings: (1) viruses; (2) tropical parasites; (3) alcohol; (4) hepatotoxic alkaloids and other food contaminants; (5) high dietary iron intakes; and (6) genetic factors.

The question will then be asked whether any of these factors can explain the gap in the prevalence of carcinoma of the liver in the Central American belt where "American kwashiorkor" is prevalent and where protein malnutrition is believed to be widespread.

Finally the question will be asked whether kwashiorkor as seen in the area of world prevalence of primary carcinoma of the liver (*i.e.*, in Africa, India, China and the Far East) can be regarded as essentially the same as "American kwashiorkor."

Does African kwashiorkor produce cirrhosis? There has been a fairly general assumption that African kwashiorkor does produce cirrhosis largely because of the assumed geographical correspondence between kwashiorkor on the one hand and of cirrhosis and primary carcinoma of the liver on the other. It must be emphasized, however, that this is only an assumption. The only real evidence on the subject that has been put on record is the opinion of Davies.[8] In discussing the pathology of the liver in kwashiorkor, he states: "The initial localization of the fat is in the periphery of the liver lobule, often sparing the cells of the lamina limitans. As the disease progresses, the fat appears nearer and nearer to the central vein until the whole lobule is full of fat. With the increase in fat, there is an infiltration of the portal triads by round cells, and fine fibrosis appears around the periphery of the lobule, spreading out in a stellate fashion from the portal triads. The fibrosis results from a thickening of the reticulum around the peripheral cells. It spreads throughout the liver, until in severe cases a state resembling a Laennec's cirrhosis is seen. If the child survives, either with or without treatment, the fat tends to disappear slowly, first from the centrolobular region, and lastly from the periphery, leaving behind a fine stellate fibrosis and round cell infiltration in the portal triads. Treatment with protein leads to rapid fat reduction and a flooding of the sinusoids with lymphocytes, but does not affect the fibrosis and cell accumulations. These may persist for many years, if not for life, and in an African appear to be a "hallmark" of kwashiorkor. Why they should be so persistent, when it is well known that in other conditions quite well-marked fibrosis of the liver can disappear, is a mystery; but it suggests that there is continuing liver damage of some sort." If this view is correct we must ask what the nature of the "continuing liver damage of some sort" referred to at the end of Davies' quotation is. Is it continuing protein malnutrition, or do other cirrhotigenic factors which will be discussed below play a part? No other definite evidence for the progressive evolution of cirrhosis of the liver out of African kwashiorkor has been recorded in the literature. The Gillmans[13] say, "We have very little information about the reactions of the livers of Africans between the ages of six and eighteen years, because during this age period, for some still undetermined reason, temporary but acute episodes of nutritional disease are rare. Biopsy in one seven-year-old child revealed that the increase in hepatic iron may commence at this age. We know most certainly that the hepatic iron content increases rapidly after the age of eighteen years. As we have indicated, primary hepatocellular carcinoma may and frequently does supervene between the ages of sixteen and twenty years and may occur at an even younger age." In other parts of the world, progressive cirrhosis of the liver does not appear to be an inevitable sequel of syndromes resembling kwashiorkor. No evidence has yet been published that the Mehlnährschaden and starch dystrophy of Europe progress to cirrhosis of the liver. In the following discussion, it will be seen that "American kwashiorkor" has not been shown to be associated geographically with a high incidence of adult liver cirrhosis. My colleagues, Doctor P. Suckling and Professor J. G. Thomson in Cape Town, have collaborated with me in searching in biopsy material for continuing evidence of cirrhosis of the liver in children recovered from kwashiorkor among the Cape colored (non-Bantu) population of Cape Town (Brock, 1949). So far, in 18 biopsies, no evidence has been found of continuing cirrhosis. In the same population group, the incidence of adult cirrhosis is no higher than it is among Cape Town Europeans.[16] In summary, therefore, it can be concluded that there is as yet no evidence that kwashiorkor inevitably leads to continuing cirrhosis of the liver after it has been cured and provided that the subsequent diet is satisfactory and cirrhotigenic agents are not prevalent. On the other hand, there is very convincing evidence that, in certain parts of Africa, at least, fibrosis may persist for many years, if not for life, after clinical recovery from kwashiorkor in the presence of "continuing liver damage of some sort"[8] or "increase in hepatic iron."[13]

Does American kwashiorkor produce cirrhosis? There seems at present to be very little, if any, evidence that American kwashiorkor produces cirrhosis. In fact, the absence in the literature of evidence for a high prevalence of primary carcinoma of the liver and of cirrhosis of the liver in the American tropical belt may be regarded as indirect evidence against such a supposition. The matter must, however, be left open until further evidence accumulates. If further accumulation of evidence should confirm that these two disorders are not prevalent, we must conclude either that "American kwashiorkor" is not the same as African kwashiorkor or that the ' continuing liver damage of some sort" postulated by Davies in Africa is not prevalent in the American tropical belt.

What other factors besides protein malnutrition enter into the production of cirrhosis of the liver? One of the few things that are certain about cirrhosis of the liver is that it may result from several, if not many, causes. In all probability, the causation is frequently multiple. I will not examine the evidence on experimental nutritional liver damage in animals since it has been so thoroughly discussed in this monograph. Turning to the human being, I do not think anybody will dispute the role of malnutrition in the production of human liver damage. What is more relevant to our present discussion is to ask: (1) Can any form of human malnutrition lead to a fibrosis which is permanent or progressive when the diet is restored to normality and when other cirrhotigenic agents are absent? (2) Can the effects of severe human malnutrition, as in kwashiorkor, be continued throughout the rest of life by milder degrees of malnutrition in the absence of other cirrhotigenic agents? (3) What other cirrhotigenic agents need be seriously considered in the causation of the cirrhosis which is so prevalent in the areas of high prevalence of primary carcinoma of the liver? The only reply that can be made to the first question is that there is, at present, no convincing evidence in the human being, and there is unlikely to be in the future. The answer must be sought by analogy from animal experiment. To the second question, the reply must be made that both animal experiment and the study of the epidemiology of kwashiorkor suggest strongly that continuing protein malnutrition may be an important factor in determining the progressive evolution of adult cirrhosis out of kwashiorkor. But if this assumption is true, why does it not also hold for the American tropical belt? In the light of this noncorrespondence, we must assume either that protein malnutrition is not continued into adolescence in the American tropical belt to the same extent or in the same form as in Africa, or else that cirrhotigenic agents other than malnutrition operate to produce adolescent cirrhosis in Africa but not in central America.

The possible or probable cirrhotigenic agents will now be discussed in order.

(*1*) *Viruses.* There can be no doubt that viruses may act as cirrhotigenic agents. It may well be argued that they act only as cirrhotigenic agents when other cirrhotigenic factors including nutritional deficiency are simultaneously operative. This view might well be supported, but the fact remains that viruses can be regarded as related to certain forms of cirrhosis in as definite a way as the tubercle bacillus may be regarded as related to tuberculosis. It is likely that viruses, if they produce progressive cirrhosis, do so because they become established in the liver over a long period of time.

(*2*) *Tropical parasites* have been blamed in the past for cirrhosis of the liver, but their etiological role seems to be far less definitely established than is that of the viruses. Schistosomiasis might be picked out as the tropical parasite for which there is most evidence of a cirrhotigenic action. The fact remains, however, that wherever schistosomiasis is prevalent, malnutrition is also prevalent; and there is no satisfactory evidence that any tropical parasite, including schistosomiasis, is more important in the evolution of tropical cirrhosis than is tropical malnutrition.

(3) *Alcohol.* Most workers accept today that "alcoholic cirrhosis" is due to a conditioned deficiency of certain nutrients. But reference to the discussion on pages 112–119 of the Ciba Foundation Symposium on Liver Disease[6] will show that there are still medical scientists, especially in France, who do not wholly accept this point of view and who, presumably, regard either the ethyl alcohol or some other constituent of alcoholic liquors as being directly hepatotoxic. Personally, I do not subscribe to this view but I think it is right that it should be brought out. For most of the area covered by Berman's map of high incidence of primary liver cancer, it is generally presumed that alcohol is either not causative or only indirectly contributive to the high incidence of the underlying cirrhosis of the liver.

(4) *Hepatotoxic alkaloids and other substances contaminating food stuffs.* There will be no question that certain alkaloids may cause necrosis of the liver and hence may lead to cirrhosis. How far can the role of this group of cirrhotigenic agents be incriminated in the production of tropical cirrhosis? The best example of the role of hepatotoxins contaminating food in the production of cirrhosis of the liver is to be found in the *Senecio* poisoning which has been described from South Africa by Selzer and Parker.[24] The disease was formerly known as bread poisoning and occurred in small family epidemics in rural areas. It has been traced to the fact that, in poor seasons, the wheat may grow to a height of only 12 to 18 inches and, in these seasons, *Senecio* is reaped with and contaminates the wheat grains with a toxic alkaloid. The illness is acute and often leads to death from necrosis of the liver. Some cases, however, recover and are left with a cirrhosis which appears to be of the postnecrotic type. Another well-known example of food contamination by hepatotoxins is the epidemic dropsy of *Argemone* poisoning.

In those regions where the effects of hepatotoxic food contaminants are known, the pathology is more in keeping with postnecrotic cirrhosis, whereas in most of the remaining areas of "nutritional liver cirrhosis," the histology is more of the Laennec type. But there is, as yet, no valid reason why the Laennec type should not result if the hepatotoxic food contaminants are consumed in small quantities over long periods of time.

In view of their unusual epidemiological and histological aspects, it is very tempting to speculate[15] upon the role of hepatotoxic alkaloids in the production of the infantile cirrhosis of India[23] and the serous hepatosis described from Jamaica by Hill.[17] This opinion is, however, only a conjecture.

(5) *Excessive intakes of iron* might well have been included under the previous paragraph but it is dealt with separately because there is difference of opinion as to whether the "siderotic cirrhosis," which is so prevalent in certain parts of Africa, is or is not due to excessive intakes of iron. The "iron overload hypothesis" of Walker[30] is backed by evidence which is either very suggestive or convincing that the daily iron intake is very high in some of the Bantu people in whom the Gillmans have described a "siderotic cirrhosis" under the term "haemochromatosis." This does not, of course, prove that the siderosis of Gillman's haemochromatosis is a result of high iron intakes. Gill-

man believes it to be the result of the metabolic disturbance which he describes in the Bantu under the name pellagra. The etiological role of high iron intakes is supported by the sporadic incidence of this siderosis in the African continent. Edington[11] has described it from the Gold Coast Colony and has associated it with high iron intakes resulting from the laterite soil of that area. Davies, on the other hand, does not find siderosis in the tropical cirrhosis of Uganda, which he believes to be a sequel of kwashiorkor and which is known to be associated with a high incidence of primary carcinoma of the liver. For those who are not familiar with Walker's work, it should be pointed out that he has demonstrated iron intakes in Bantu subjects rising to figures between 100 and 200 mgm. of iron per day; that is ten times or more the intake of iron in the average Western European diet.

(*6*) *Genetic influences.* Few people will deny that genetic influences may play a role in the prevalence of primary carcinoma of the liver in certain parts of the world. But it is very doubtful whether this influence is at all important. Certainly, it cannot be accepted until environmental factors have been excluded. The world regions of high prevalence of primary carcinoma of the liver are regions of pigmented people, and it is possible that pigmented people have a metabolism more conducive to the development of liver cancer. On the other hand, the area is also an area of tropical malnutrition and tropical parasites, and one or both of these is likely to be more important etiologically. It is interesting that, in those parts of the world where pigmented people live outside the tropical belt, there is little or no evidence of increased prevalence of primary carcinoma of the liver. The Indians of North America may be cited as examples. China produces interesting negative evidence because, as pointed out earlier, it is probably only those Chinese who live in, or close to, the tropical belt who show a high incidence. On the other hand, the reports of prevalence of primary carcinoma of the liver in Chinese people living in the United States of America might be evidence for the genetic factor, if it could be shown that these Chinese were born in the United States and had not migrated from Southern China. The reports are not clear on this point. The Japanese are interesting because they are a pigmented people living outside the tropical belt who show an incidence of primary carcinoma of the liver intermediate between the tropical world and that of Northern Europe and America. Consideration of this factor can, however, be regarded at the present time as merely speculative.

Can any of these cirrhotigenic agents explain the central American gap on the world map of high prevalence of carcinoma of the liver and the apparent infrequency of cirrhosis? Certainly any, or several, of these cirrhotigenic agents could explain the central American gap on the world map since they might constitute the "continuing liver damage of some sort" postulated by Davies in discussing the development of fibrosis of the liver as a sequel to kwashiorkor. At present, there is little or no evidence to suggest which of these factors might be important in explaining the area of noncorrespondence on the map. But the careful investigation of all of them might well throw light on the etiology and pathogenesis of many varieties of liver damage.

The concept that malnutrition, and particularly protein malnutrition, is important in the genesis of human liver cirrhosis emerges from this discussion as highly probable. Malnutrition appears, however, not to be adequate to explain the peculiar world distribution of the prevalence of severe liver damage in adults. It is clear that considerable attention has still to be given to the role of other cirrhotigenic agents in etiology and pathogenesis. Nor does the discussion seriously challenge the assumption that "American kwashiorkor" is fundamentally similar to kwashiorkor in Africa. If it is established by further collection of data that American kwashiorkor is not, in fact, geographically associated with a high incidence of adult liver damage as is the case in the African continent, the difference may still be explained by a greater severity and duration of continuing protein malnutrition and by the coexistence of other cirrhotigenic agents in the evolution of the prevalent adult liver damage in the African continent.

The transactions of the Sixth Conference on Liver Injury arranged by the Josiah Macy, Jr., Foundation showed a fair degree of agreement that pathologists can distinguish between Laennec's cirrhosis and postnecrotic (toxic) cirrhosis, but the agreement is by no means unanimous. Can the morbid histologist predict from the histological pattern of cirrhosis in areas of high incidence whether the etiology has been wholly or partly nutritional or what the nature of other cirrhotigenic agents may have been? The answer is probably "No." The clues must, therefore, still be sought in a study of the epidemiology of the diseases under discussion, and their relation to prevailing dietary patterns and other possible etiologic mechanisms. The experimentalists can develop the clues in animal studies, but they must complicate their experiments by studying the combined effects of dietary deficiency and of other cirrhotigenic agents acting over long periods of time in small doses.

Summary

(*1*) Kwashiorkor is defined and described as it occurs in the African continent. It is concluded that, although there are many clinical patterns or variants of the syndrome, differing probably according to the particular pattern of amino acid and vitamin deficiency supplied by local diets, yet the syndrome is basically an entity from the point of view of etiology, pathogenesis, and pathology.

(*2*) It is concluded that its causation is "protein malnutrition" in the sense defined by the Third Session of the Joint F. A. O./W. H. O. Committee on Nutrition.

(*3*) It is concluded that present evidence suggests that related syndromes described under many names from different parts of the world are probably essentially the same as kwashiorkor in etiology, pathogenesis, and pathology, and that the same may be said of Mehlnährschaden and starch dystrophy except in so far as the clinical picture is modified in these latter two syndromes by the relative absence of pigment in the skin.

(*4*) The world incidence of kwashiorkor and related "protein malnutrition" syndromes is compared with the areas where there is a high incidence of "adult cirrhosis" and primary carcinoma of the liver. It is concluded that there is enough correspondence to suggest some etiological relationships between kwashiorkor and these two conditions, but that there are also important areas of noncorrespondence.

(*5*) It is felt that further investigation of the areas of noncorrespondence might throw light upon the relations between fatty infiltration, necrosis, and different patterns of cirrhosis in the liver, as well as upon the origin of siderotic cirrhosis.

(*6*) It is concluded that at the present stage of our knowledge it can be assumed that dietary deficiency plays a large part in the etiology of the adult cirrhosis which, in areas of high incidence, is correlated with a high incidence of primary carcinoma of the liver. The etiological role of tropical parasitism, although apparently not a major factor, cannot be entirely ignored. More attention should be devoted to the possibility of chronic contamination of food with hepatotoxic alkaloids as a factor in the etiology of so-called "nutritional cirrhosis."

(*7*) The possibility of adult "nutritional cirrhosis" arising from kwashiorkor as a direct and necessary sequel must be regarded as unlikely, but the prevalent dietary pattern which produces kwashiorkor in some populations in Africa must be regarded as being at least one of the causes of high prevalence of adult cirrhosis and primary carcinoma of the liver in the same continent.

(*8*) Kwashiorkor of the African continent appears to be very similar to, and probably is basically the same in etiology and pathogenesis as, its equivalents described here as "American kwashiorkor." But this conclusion cannot be finally accepted until some explanation is given for the apparent low incidence of adult cirrhosis and primary carcinoma of the liver in the American tropics.

*Appendix—Definitions**

Cirrhosis. This term is used in the wide sense accepted by the group of eight pathologists under the chairmanship of Doctor Harry Goldblatt.[14] No attempt is made in discussing the epidemiology of cirrhosis to use such terms as "fibrosis,"[18b] "elastosis," and "pseudocirrhosis."[29] Reference is made to the distinction between Laennec and postnecrotic (toxic) varieties[14] without attempting to apply it to the discussion. A number of qualifying adjectives are attached at times to the term "cirrhosis." Most of these are used in a purely descriptive sense. "Adult" cirrhosis is used solely with reference to the age of the patient. "Tropical" cirrhosis is used to signify the cirrhosis which is prevalent in tropical countries without any etiological connotation. "Nutritional" cirrhosis is used when it is believed that dietary deficiency plays an important part in etiology, but the term does not exclude the possibility of etiological factors other than diet. In this usage, "tropical" and "nutritional" cirrhosis are almost interchangeable terms. The term "siderotic" cirrhosis has deliberately been used to signify cirrhosis with considerable increase in iron content of the total liver. This has been done so as to avoid confusion with terms such as "haemochromatosis" and "pigmentary cirrhosis." The term was chosen because it is purely descriptive and does not judge between etiological theories such as Walker's "iron overload" hypothesis and the Gillmans' "cytosiderosis" concept. It is felt that the use of the term "haemochromatosis" by Strachan[27] and by the Gillmans[13] for the siderotic cirrhosis of the Bantu people of southern Africa is confusing since the balance of evidence suggests that these cases are quite distinct from classical haemochromatosis.[25]

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* This Appendix was added by the author in presenting his paper for publication after the conference.

Primary liver cancer or primary carcinoma of the liver. These terms are used interchangeably and are preferred to "hepatoma" and "hepatocellular carcinoma" in this communication because the former terms are wider and the precise cellular type of carcinoma is not always specified in reports from the areas of prevalence. In all probability, however, the prevalent condition is usually a hepatocellular carcinoma.

Pellagra. The term "infantile pellagra" has been specifically rejected in favor of kwashiorkor, and a few words should be said about its use in relation to the prevalent clinical syndrome in adult Bantu subjects in South Africa which is linked by the Gillmans[13] with the siderotic cirrhosis which they call haemochromatosis. They do not define the "pellagra" they are describing except in the negative sense: "We are of the opinion that, unless the dermatosis is present, then the nutritional syndrome should not be regarded as pellagra." They clearly recognize the possibility that pellagrous dermatosis may occur in several different patterns of complex metabolic disturbance resulting from different dietary patterns, but use the term indiscriminately for them all. We see, in Cape Town, pellagra in Cape colored people, of mixed but predominantly non-Bantu blood,[4] which is indistinguishable from the classical pellagra described in Southern Europe and the Southern U. S. A. We see also "pellagra" in Bantu subjects which is apparently indistinguishable from the "pellagra" described by the Gillmans. In our experience, the disease in these two different races is very different and it is confusing to call them both pellagra. I prefer to call the latter variety "Bantu malnutrition with pellagrous dermatosis." At least, it ought to be called "Bantu pellagra" to distinguish it from the classical type. To mention only a few of the differences, pellagra in the Cape colored people is not associated with siderotic cirrhosis nor with any increased susceptibility to primary carcinoma of the liver. The same is true of classical pellagra in Southern Europe and Southern U. S. A. "Bantu pellagrins" in South Africa, on the other hand, have a high incidence of siderotic cirrhosis and primary liver cancer.

Necrosis. Experimentally, fatty liver cirrhosis and acute necrosis of the liver appear to be quite distinct processes as is evidenced by the division of the proceedings of this conference between these two headings. There has been a tendency among experimentalists in dietary liver damage to confine the term "necrosis" to the acute necrotic variety instead of using it in its wider meaning of "death of cells" from any cause. This trend may be useful although it is etymologically dubious. It is necessary, therefore, to emphasize that Brock and Autret in their report used the term in its wide sense and did not imply thereby any necessary connection with the experimental lesion which is discussed in this monograph under the heading "liver necrosis" which might better have been written "acute liver necrosis." The available evidence in regard to kwashiorkor undoubtedly suggests close analogies with experimental fatty liver cirrhosis, but the analogy cannot be pushed too far. This is particularly important in respect to adult "nutritional cirrhosis." In discussing this subject Himsworth[18] says "massive hepatic necrosis and its sequelae are unusually common in tropical and subtropical races subject to malnutrition." The factors, other than diet, which may be involved in the production of tropical cirrhosis are categorized in the text. Some of these are known to cause acute necrosis of the liver.

Postscript

After hearing the discussion at the conference on which this monograph is based, I think it is necessary for me to emphasize that my personal experience of kwashiorkor is limited to the African continent, but includes the material seen in Johannesburg and Pretoria where the syndrome is seen in Bantu children who have usually not been exposed to tropical parasites, and in Cape Town where among Cape colored children there has been no opportunity for exposure to tropical parasites.

With regard to the comparison with "American kwashiorkor," I am handicapped by the fact that I have not yet seen the report by Autret and Behar. My impression from the published literature and from pictures shown is that ' American kwashiorkor" at least in cities is often more dominated by undernutrition than is the case in what has been defined as "protein malnutrition," especially as exemplified by the pictures in the monograph by Brock and Autret. Obviously, there are in all areas all degrees of transition between protein malnutrition and undernutrition.

Conversations at the conference confirm my impression that cirrhosis and primary carcinoma of the liver are far more prevalent in the American tropical belt than the literature indicates.

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Discussion of the Paper

Doctor Schwarz: I wish to congratulate Dr. Brock on his very outstanding review and on the remarkable achievements which he, in collaboration with other members of the Joint F. A. O/W. H. O. Expert Committee on Nutrition, has accomplished. May I be permitted, however, to remark, that to my knowledge there is no conclusive evidence that kwashiorkor is just a protein deficiency. I may be wrong, but it seems advisable to sound a warning against the general acceptance of this idea. For *practical* purposes, particularly for indoctrinating and training less educated populaces, the designation of kwashiorkor as a "protein" deficiency might be of value. This, however, should not distract the attention of a scientific forum from the fact that the therapeutic benefit derived in a deficiency disease from protein foods or even from an (impure) protein itself, like casein, does not necessarily indicate that this disease is caused by lack of "protein" as such. The effect can equally well be due to trace factors contained in these proteinaceous materials, or also from one individual protein building stone. The quantitative separation of vitamins from proteins is extremely difficult. Finding or preparing vitamin-free proteins for vitamin-free diets has been one of the most serious obstacles in vitamin research during the last 50 years.

We know that, with the exception of fat soluble vitamins, all important dietary factors may occur in nature in more or less firmly bound forms. In many cases autolytic enzymes are required for their liberation and in some instances, for example in the case of biotin, hydrolysis with strong acid must be used to achieve complete release. Still fresh in our memory is vitamin B_{12} which not long ago was called "animal protein factor." This monograph contains two further examples of specific nutritional factors which occur strongly bound to protein. Both of these concern the liver: The xanthine oxidase factor, which after prolonged attempts at purification and isolation from plant proteins has now been disclosed to be a trace element, molybdenum,[1] and Factor 3 against dietary necrotic liver degeneration, which is obtained in concentrated form from casein and which is present even in so-called vitamin-free caseins.[2]

Taking these observations into account and realizing further that not all of the factors protecting against fatty liver and cirrhosis have been identified as yet, I think it would be fair to say that one should be reluctant to label kwashiorkor as "protein deficiency," at least as long as it has not been shown to be cured by pure amino acid mixtures.

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THE PATHOLOGY OF DIETARY LIVER DISEASE IN TROPICAL AFRICA

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The only form of dietary liver disease in tropical Africa about which it is possible to speak with any precision is the extreme fatty infiltration which is seen in kwashiorkor. In a florid case of this disease (*e.g.*, in a child of about two years of age), the liver, while not very much enlarged, is a tawny yellow or buff color or, in a very anemic child, may be stone colored, due to intense fatty infiltration. In older children, some degree of increased fibrosis of the liver may be appreciated. Not all children with kwashiorkor have such a degree of fatty infiltration, for in some there may be little or no fat.

The changes in the liver in kwashiorkor, as shown by biopsy and autopsy studies, go through a sequence which results in permanent alterations to the structure of the liver. In West Africa, attention has been drawn (Auffret and Tanguy, 1950; Silveira and Jelliffe, 1952) to the presence of gross fatty infiltration of the liver in very young children. Such lesions are not commonly encountered among young children in Uganda and, where there is any appreciable degree of fatty change seen in the infant liver, it is usually centrolobular in distribution. So far as my experience goes, the livers of new-born African children and of neonates in Uganda do not differ appreciably from the livers of European children. Apart, therefore, from the presence of malaria pigment, which after a while acts as a convenient delineator of the boundaries between liver lobules, the African liver in those first few months of life, when the child is growing well (Welbourn, 1951), presents no significant change. As at the weaning period, the weight curve begins to lag and the clinical condition begins to deteriorate, the liver cells become hypocytoplasmic and are filled with a fine watery vacuolation, and fat droplets begin to appear, always being visible first at the periphery of the liver lobules. This is always the case in children where there is no precipitating factor; *e.g.*, tuberculosis. In complicated cases, the initial fat deposition may be centrolobular or irregularly sited, varying from lobule to lobule. Where this has been recognized by a study of biopsy material, the attention of clinicians has, on several occasions, been drawn to the presence of a hitherto unrecognized infective condition (Davies, 1948, 1950).

Provided there is no such complicating factor, the initial locus of the fat deposition is in the cells at the periphery of the liver lobule, though the cells of the lamina limitans round the portal triads often remain free of fat for a considerable time. As the clinical condition deteriorates, the peripheral fat droplets increase in size and the cells down the sinusoids toward the central vein are progressively involved till, in a severe case, practically every cell is full of fat and some small fatty microcysts may be seen. FIGURE 1 shows a part of two liver lobules with a portal triad and two central veins with fatty infiltration of the liver cells, the larger lobules of fat being in the outer parts of the lobule. Dark malaria pigment is seen also, collected chiefly at the periphery

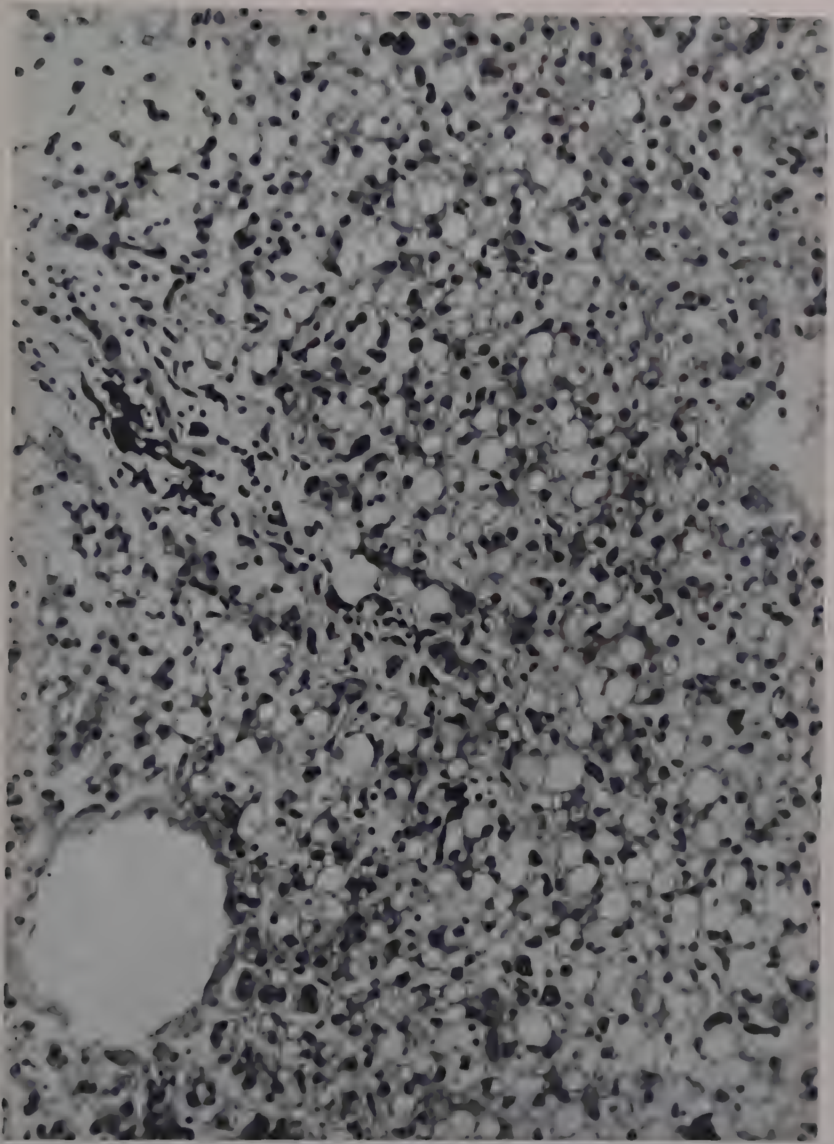

FIGURE 1. Liver of child with kwashiorkor, showing the fatty infiltration in the peripheral regions where some liver cells are unaffected. Malaria pigment is present in the portal triad. Two dilated central veins are seen. H & E stain. ×210.

with considerable masses in the portal triad. Some of the peripheral cells are free of fat. It will be noted that quite a number of cells are visible in the sinusoids and these are chiefly lymphocytes which appear in some obscure way to be concerned with the infiltration or removal of fat, because, when either of these processes is active, they occur in great numbers in the sinusoids.

With the infiltration of the fat into the liver, certain other changes are noticeable. The lymphocytic infiltration of the sinusoids has been mentioned, but these cells and many others become concentrated in the portal triads. The most plentiful cells are lymphocytes, but plasma cells and macrophages and neutrophil and eosinophil polymorphonuclear cells may also be present, the latter two types being plentiful in cases complicated by a septic infection or ancylostomiasis.

Such notable collections of cells in the portal triads are seen in FIGURE 2. As you can see, there is a considerable degree of fatty infiltration with the most intense fatty deposition in the peripheral cells of the liver lobule. The portal triads are crammed with cells and they are also present to some extent along the periphery of the lobule. The cellular infiltration of the portal triads seems to be one of the persisting alterations in the liver because, except in the early months of life, it is very rare to see an African liver which does not contain an excessive number of cells in the portal triads. In addition, comma-shaped nuclei are present in fair numbers in the portal triads, and these are probably the nuclei of fibrioblasts. They do not seem to occur, in the early stages at least, outside the portal triads.

The third change that takes place is in the reticulin framework in the periphery of the lobule. The fibres thicken and split and reduplicate. This change takes place round the peripheral cells and is most marked at an early stage near the portal triads, but later it is apparent round the whole of the periphery of the lobule. The lamina limitans cells are separated by the thickening reticulin and they and the peripheral cells appear to undergo a process of strangulation for some of them disappear with collapse of the reticulin framework. The reticulin undergoes collagenization; so at an early stage, the lobular outline is accentuated not only by the malaria pigment, but also by the reticulin thickening and collagenization. FIGURE 3 shows such fibrotic changes. The liver is moderately fatty, the portal triads are being joined one to another by the reticulin thickening and collagenization. This accentuation of the lobular pattern of the liver is never so marked in children we have studied that it could be called a cirrhosis, and these changes all occur round the lobule periphery. In a few cases, the collagenization is so marked that all the portal triads are linked together by fine strands of fibrous tissue, more reminiscent of the liver of a pig than a normal human liver. In his studies of the fatty liver in cases with tuberculosis, Saphir (1929) remarked on the development in a small percentage of his cases of such a true unilobular type of fibrosis.

In time, should the child survive even without treatment by a high-protein diet, the fat will diminish and slowly disappear from the liver even without any marked clinical improvement; and it can be removed more speedily by feeding a high-protein diet. Under both circumstances, the fat diminishes and disappears first from the centrolobular regions and last of all from the peripheral cells. As it leaves, the fibrosis often seems to increase and become more apparent. The cells, after the fat has left, exhibit the same hypocytoplasmic appearance as before it appeared. Though the reticulin thickening and fibrosis seem to start with the fatty infiltration, the relationship is certainly not simple. It is a time relationship rather than a causal relationship.

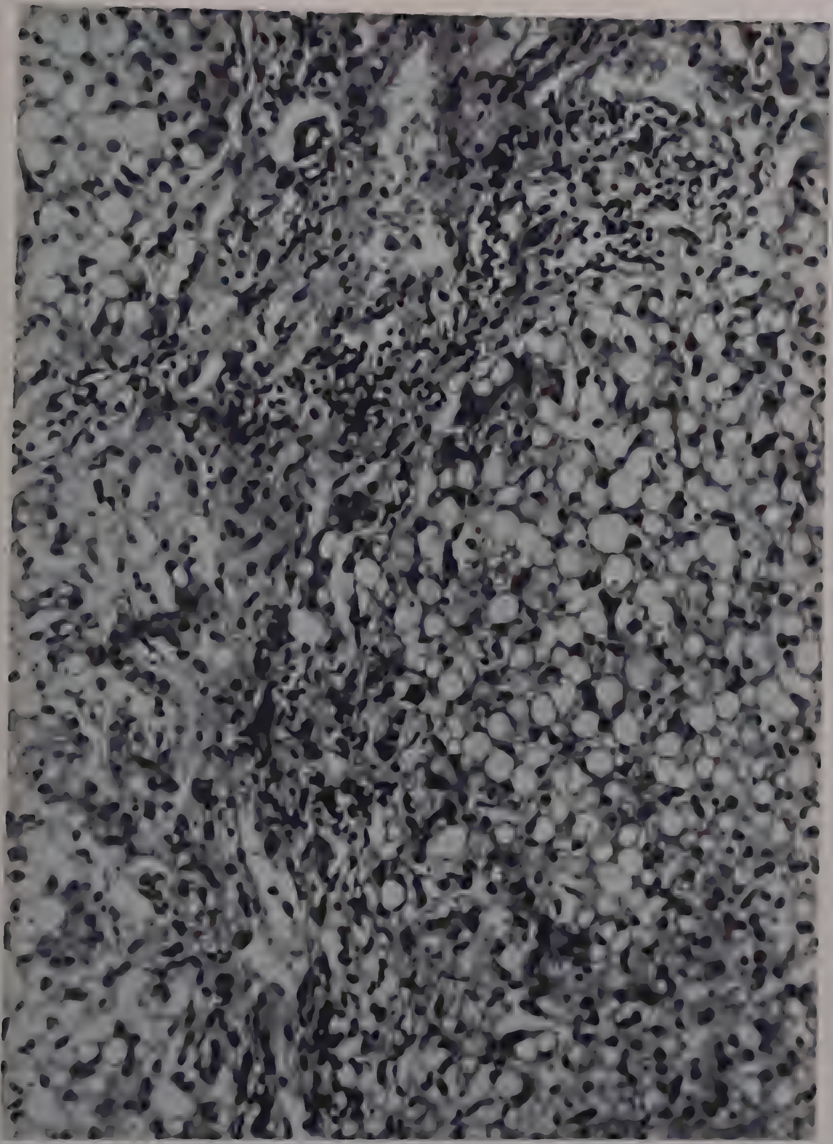

FIGURE 2. Liver of child with kwashiorkor, showing fibrotic process and cell infiltration at the periphery of the lobule where the fat infiltration is maximal. H & E stain. ×210.

Sometimes there is severe fat infiltration with minimal fibrosis; sometimes marked fibrosis with little fat infiltration; and the cause of the fibrosis awaits elucidation, particularly as Waterlow (1951) has shown in West Africa that a similar type of fibrosis can develop in the liver without fatty infiltration. Our experience leads us to suppose that there are at least two sets of causal factors

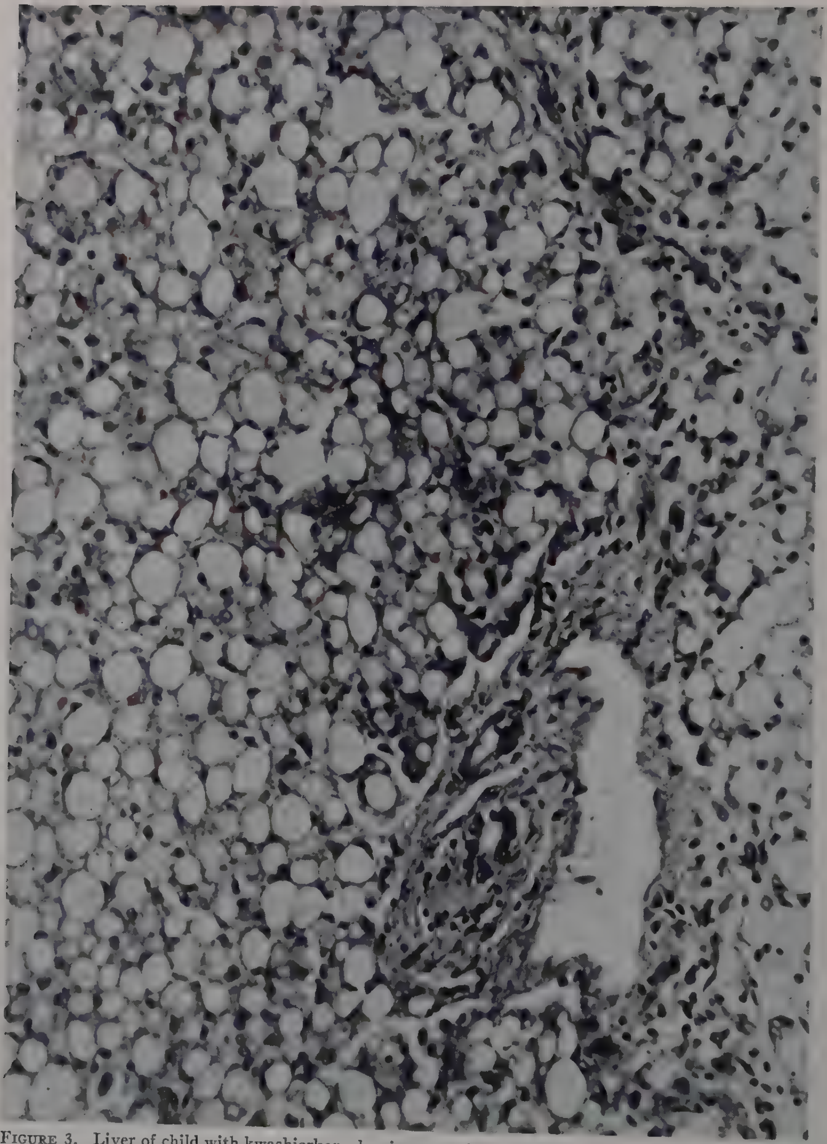

FIGURE 3. Liver of child with kwashiorkor, showing gross fatty infiltrations. The early periportal fibrosis spreading from the portal triad can be seen. H & E stain. ×210.

operating, one leading to the fatty infiltration, the other to the fibrogenesis. The net result is, however, that the vast majority of Africans, both adults and children after the age of two years, have livers with considerable cell infiltrations in the portal triads, with scarring in the portal areas, scars radiating from the portal triads with strands of fibrous tissue which tend to join one triad to

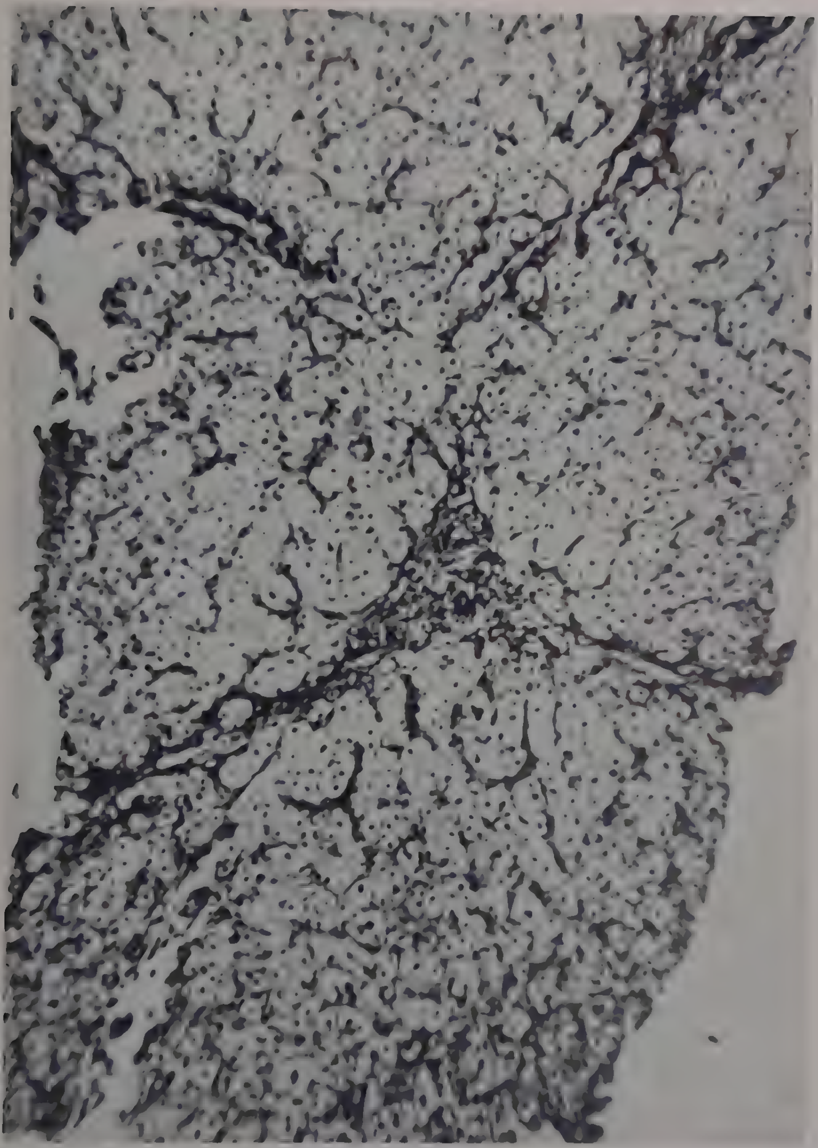

FIGURE 4. Biopsy specimen of African child's liver showing stellate fibrosis of the portal triad extending to delineate the lobular boundaries. Reticulin stain. ×150.

the next along the boundary of the lobule. FIGURE 4, taken from a liver biopsy, shows this lesion which I call a "stellate fibrosis." The smooth outline of the portal triad and the lamina limitans are disrupted by these fibrous scars in the vast majority of African livers. I once suggested (Davies, 1948) that these lesions were the "hallmarks" of kwashiorkor. In Uganda, they are not found before the weaning period and they are almost universal afterwards; so that some changes take place in this period that mark the period if not the disease.

It would, I think, be most unwise for me to speculate on the possible causes of the fibrosis. I do not think the fibrosis is a result of the fatty change, but whatever the cause, the stellate fibrosis is a persistent lesion and I think it continues in a slow, stealthy fashion to increase in the absence of fat from the liver. Nor have we any very clear ideas on the exact causes of the fatty infiltration except a very strong suspicion that it is somehow linked to the atrophy of the pancreatic acinar cells and the other cells which lose protein by enzyme secretion. The histological evidence we have accumulated suggests that the pancreatic atrophy precedes the liver changes and that the fatty infiltration of the liver is a transient though important episode in the disease. The primary involvement of the pancreas has also been shown by Véghelyi (1951). But the precise connections between the pancreatic atrophy and the fatty infiltration of the liver have not been discovered, and to discuss them would take me outside my field.

It is more important to discuss the relationship of the changes in kwashiorkor to disease of the liver in later life. We are most ignorant about the condition of the liver in the later years of African childhood and the earlier years of adolescence. But during this period, and indeed for the rest of his life, the African is eating a diet qualitatively little better than that on which he developed kwashiorkor. A diet low in protein, especially animal protein, and high in carbohydrate is all too common. Yet cirrhosis of the liver is rarely seen in childhood in Uganda nor in the early years of adolescence. Puberty with its growth spurt does not bring out a fresh crop of kwashiorkor cases and we do not know why this is so. Perhaps puberty in Africans is more delayed and the growth spurt not so marked. We know that the onset of menstruation is delayed. Moreover, we must remember that the endocrine changes in kwashiorkor, though undoubtedly important, have not yet been studied. But in the late teens and in adult life, a condition occurs pathologically and, in many respects clinically, identical with kwashiorkor as seen in children. Indeed, much early work on this disease was largely devoted to the study of adult cases. The atrophy of the pancreas and of the intestines is just as marked and there is the same fatty infiltration of the liver though this is not so extreme. The major difference lies in the much greater amount of fibrous tissue present in the liver, a considerable increase in the stellate fibrosis. In some cases, irregular strands of fibrous tissue cut off groups of cells at the periphery of the liver lobules. Both clinically and pathologically, some cases merge into Laennec cirrhosis, but the connection between these states remains obscure. The gross fatty infiltration seen in children is not very frequently encountered in adults and we have no evidence to suggest that our cases of Laennec cirrhosis have gone through a long continued stage of fatty infiltration in adolescence. Iron pigment is not present in unusual amounts in our cases and the only pigment seen is a brown perinuclear pigmentation apparently due to a lipochromic substance.

Laennec cirrhosis is quite frequently encountered in Africans. Among 2718 autopsies there were 115 cases as well as 74 cases of postnecrotic scarring. Analysis of these cases has shown (Davies, 1952) that, contrary to expectations, there is no great sex difference in frequency of cirrhosis of the liver, nor in the frequency of primary liver carcinoma, nine cholangiomas and 33 hepatocellular carcinomas being encountered. We suspect that the great frequency of cirrhosis and carcinoma of the liver are in some ways linked to malnutrition and probably to protein malnutrition.

I shall, if I may, conclude this brief presentation by summarizing our main problems as I see them. The frequency and importance of kwashiorkor is now well recognized. The basic lesion seems to be a selective atrophy of the enzyme-secreting glands affecting the pancreas, the small intestines, and the salivary and lacrimal glands. We do not know the precise cause of this atrophy. At an early stage in the disease, there is an intense but transient fatty infiltration of the liver, the fat droplets occurring first in the peripheral cells of the liver lobule, which are the last to be freed of fat. We do not know the cause of this fat infiltration, its relation to the preceding enzyme cell atrophy, or the factors that enhance its removal from the liver. With the fatty infiltration, there occurs a flooding of the portal triads and to a lesser extent the sinusoids with inflammatory cells, the lymphocytes being especially prominent. We do not know what they are doing. There is also the reticular thickening and the fibrotic process near the portal triads and around the lobule periphery. The cause of this awaits elucidation. We do not know how this continues to progress in the absence of fat from the liver, the significance of this progression, its extent, or its relation to Laennec cirrhosis. We do not know the cause of the great frequency of Laennec cirrhosis, but we do not think that it is a sequel of infective hepatitis. We also do not know the causal factors of carcinoma of the liver although we suspect them to be endocrinal. Malnutrition, liver disease, and hormonal alterations seem to be fundamental to our understanding of disease in Africans. They are being investigated by only a few widely-scattered scientists.

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CLINICAL ASPECTS OF THE TREATMENT OF KWASHIORKOR*

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Terminology and Etiology

Although this paper discusses the clinical aspects of kwashiorkor, it is first of all necessary to discuss briefly etiology, for this determines the approach to the problem of therapy. There is now general agreement that a distinct clinical and pathological disease-process, called kwashiorkor, is very common in certain tropical countries. This condition occurs mainly in young children and follows the prolonged consumption of diets which have lacked variety and in which the proportion of protein, especially that of animal origin, has been low, whereas the proportion of carbohydrate has been high. These diets always have rather a low protein-calorie ratio and the animal protein-calorie ratio is always very low. It is considered that this unbalanced diet was usually given in amounts sufficient to satisfy the child's hunger, but more information is desired on this important point. At a certain stage in the illness, appetite decreases and anorexia begins, so that a decreased intake of this unbalanced diet often then occurs (Welbourn, 1953). At this stage of the illness, there is also impaired digestion and utilization of all foods. The intake of fats is also usually low. Children who develop kwashiorkor have been receiving little or no milk, either of human or animal origin, and very little protein of animal origin. The condition is therefore treated, cured, and prevented by giving diets rich in protein. I should also make it clear that certain of my colleagues in Uganda consider that older children and adults may occasionally suffer from a similar or an allied disorder, but this paper is confined to a study of the disease in young children, among whom the condition is far more common because of their high requirements for protein and allied substances.

Kwashiorkor is one member of a big group of diseases referred to as the diseases of protein malnutrition. It would appear unwise to speak of kwashiorkor as merely "protein deficiency" for there are many varieties of protein deficiency even among children. For instance, one type of protein deficiency occurs in nephrosis, and it is quite distinct from kwashiorkor. In addition, it is quite uncertain how far calorie intake, total protein intake, one or more amino-acids, or vitamins are responsible, as well as other substances, known or unknown, but concerned with animal protein or with protein metabolism. It is also not known why an adequate supply of human breast milk, which has rather the low protein-calorie ratio of 1.5 gm. to 100 calories is so highly protective against kwashiorkor (György, 1952).

A noncommittal term, such as kwashiorkor (Williams, 1933), is therefore preferred. In many areas of Africa, in which there is little cows' milk and few protein-rich cereals, the peasants have noticed that healthy, breast-fed babies often develop a serious illness shortly after complete weaning. In many tropical countries, as in Europe until Elizabethan times, breast feeding may

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* Thanks are due to the Director of Medical Services, Uganda, for permission to publish this paper.

continue for two or three years and often with considerable benefit. This was often possible in Africa under the conditions of marital life which were a part of polygamy, but conditions and customs are changing rapidly. If a second pregnancy occurred prematurely, the first child was weaned and became ill. The second pregnancy was considered to kill the first child, and the disease often carried a stigma. This illness was called kwashiorkor among the Ga people of the Gold Coast, among whom Doctor Cicely Williams was working when she published her accounts of this disease (1933). Other tribes have other names and about fifty different terms have been employed by different doctors. These names often refer unfortunately to multiple deficiencies, possibly those of the vitamins, and these terms have crept into the literature, especially that of South America. Yet treating these cases mainly by multiple-vitamin therapy often results in fatalities.

Recently it has become clear that Czerny & Keller (1906) were describing an identical condition in Germany under the name of Mehlnährschaden. Their description of the disease, however, was very incomplete. No changes were noted in the pigment of the hair and the pancreatic lesion was not described; so that subsequent workers, such as Véghelyi (1948, 1950), who almost rediscovered the condition in Hungary and called it "nutritional edema," were unable to identify this disease with Mehlnährschaden. The old and correct teaching of the German pediatricians was kept alive and developed in Italy, where Frontali (1952) has confirmed the presence of the pancreatic lesion and the hair changes in this disease (called "starchy food dystrophy" in that country). This disease is present in nearly 5 per cent of children in Southern Italy. The presence of the characteristic "enamel-paint dermatosis" and "flexural fissures" of kwashiorkor has however not been described in any case of Mehlnährschaden, or "starchy food dystropy."

In the literature of the United States, a few cases of kwashiorkor probably have been described, such as that of McEnery (1933). It is difficult to evaluate the more numerous reports on the cases of "nutritional edema" seen in young children in the United States and in other countries. In cases of "nutritional edema" in infancy, there has not been described the dermatosis, the fissures, the dyspigmentation, the pancreatic lesion, or the high mortality, which are characteristic features of kwashiorkor. "Nutritional edema" was ascribed at one time to protein deficiency and it is probable that many cases of this disease, especially among young children, were cases of kwashiorkor, and were not cases of hunger edema due to calorie deficiency, or beri-beri due to thiamine deficiency, all three of which are different varieties of "nutritional edema." The latter is not a suitable term for kwashiorkor, for some severe cases show no edema and mild cases of kwashiorkor never show any edema.

It is proposed to confine the attention to clinical aspects of the treatment of cases of severe kwashiorkor in childhood, since little is known concerning that of the milder state. There is fairly general agreement that mild kwashiorkor is probably very common in many tropical countries. It is, however, difficult to define (FIGURE 1). Thus, Welbourn (1953) has found signs suggestive of mild kwashiorkor in 46 per cent of the children attending child welfare clinics near Kampala, Uganda. Wherever possible, treatment and prevention of the milder disease should be by vegetable protein, for this is cheaper and more plentiful.

THE CLINICAL PICTURE OF A TYPICAL CASE OF SEVERE KWASHIORKOR

This is so distinctive (Trowell, Davies, & Dean, 1952) that a diagnosis can usually be made as soon as the case is seen (FIGURES 2 and 3). The child is seriously ill and is usually from 9 to 36 months of age, although typical cases can occur outside this range. The weight and height are always very subnormal. Mental apathy is always present and peevishness on being disturbed is usually noted. The appetite in advanced cases is always very poor. The abdomen is usually distended. Undigested food is passed in bulky or loose stools, which are usually frequent. Diarrhea may be very severe or absent, and depends on the food eaten. Subcutaneous fat is often well preserved during the early stages, but is lost later if life is prolonged. The muscles waste early and teverely in this disorder. Edema is usually present, but may be absent or slight in cases otherwise typical. Edema tends to be generalized, the feet and face being involved early. Effusions into serous sacs are absent or slight so that tapping is never required. This edema is not explained by cardiac failure or by renal disease, although the urine may contain a trace of albumin. Casts and cells are, however, absent or extremely scanty. The hair always shows some change in its texture, becoming soft and straight. It is easily shed or pulled out of the scalp. Some change and loss of hair pigment is usually, but not always, seen; but this change may only be indisputable in those whose hair previously was black. Dyspigmentation, however, occurs in all races, whatever the color of the hair. Some change in skin pigmentation may occur. Generalized hypopigmentation, especially obvious around the mouth, may be seen in Africans. Races with a white skin usually show no change but generalized hyperpigmentation has been occasionally described.

The various eruptions on the skin are complex and should not attract too much attention. Like the changes in the mucous membranes, they are essentially variable. Usually they are only seen as a terminal condition in very advanced cases. They are not present in all cases, even at death. The only dermatosis which, in my opinion, is peculiar to kwashiorkor is best described as "enamel-paint dermatosis" (FIGURE 4), or "erosive dermatosis," since, although originally likened to the "crazy-pavement" of an old English garden path, this is not a happy comparison and the latter term has been used to describe several different skin lesions. "Enamel-paint dermatosis" appears almost exclusively on areas exposed to much pressure, especially if this is combined with moisture, any discharge, or the sweat which is common in the tropics. This dermatosis has a definite evolution. At first, small purple stains darken rapidly into hyperpigmented, raised, burnished plaques and these appear first in the inguinal and napkin areas and over the bony prominences of the legs, trunk, arms, and head (FIGURE 5). These plaques have a sharply defined linear edge. They enlarge rapidly and coalesce, then proceed to loosen, as with some underlying moisture, and peel, thus disclosing pale, eroded, moist areas of raw skin. These plaques never appear in response to sunlight, although in many respects they resemble the eruption seen in certain

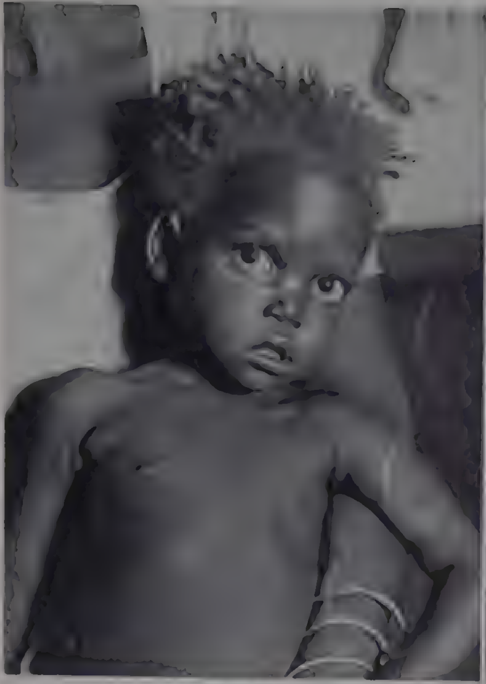

FIGURE 1

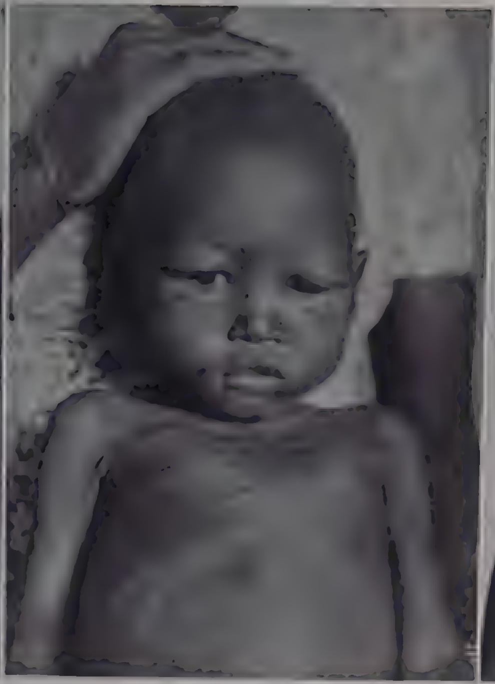

FIGURE 2

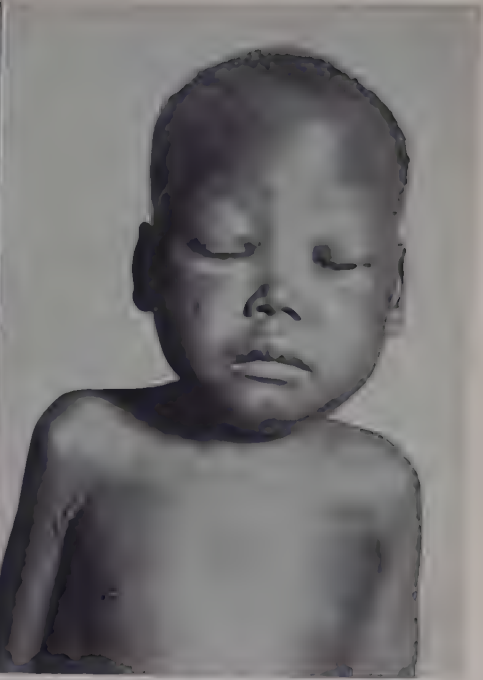

FIGURE 3

FIGURE 1 (left). African child with mild kwashiorkor, underweight, but nutritional status [illegible] loss of hair pigmentation. Good [illegible] of yellow and black [illegible] hair appeared after many weeks on skim milk. FIGURE 2 (center). African child with severe [illegible], marked wasting, edema, depigmented hair, and fissured corner of mouth. FIGURE 3 (right). African child with severe kwashiorkor, showing depigmented hair and skin; also edema.

cases of acute pellagra. At times, erosions may occur in any of these areas, with little in the way of preceding plaques. These erosions are easily dismissed as areas of intertrigo, which indeed they are. At the same time, in a few advanced cases, very deep fissures occur in the flexures, especially if sustained flexion of a limb causes pressure on the crease in the skin. "Flexural fissures," therefore, appear in the armpit, front of the elbow, back of the knee, and behind the pinna (FIGURE 6), and at the angle of the mouth, where they resemble angular stomatitis, but they are deep fissures which bleed and never appear sodden. "Enamel-paint dermatosis," "erosive dermatosis," and "flexural fissures" have not been described, as far as I am aware, in any other disease. All commence healing within a few days, if not hours, after giving skim milk protein.

Large areas of glazed and "crackled skin" may occur in areas exposed to irritation and to dryness. This skin shows little tendency to desquamate spontaneously in large plaques unless the condition is very extensive and is subsequently well treated. This condition, often called "mosaic skin" or "crackled skin," would appear to be largely a nonspecific sign and may occur in many states of undernutrition and responds to an increase in the diet. In my opinion, these conditions tend to be more severe in any disease of protein malnutrition than in states of undernutrition.

In addition, a pellagrous eruption, even with vesiculation, may occur at the periphery of the limbs on areas exposed to sunlight and to wind. It is most common in children taking much maize, but it can occur on other diets. This dermatosis only occurs as a terminal manifestation a few days or weeks before death. The eruption is seen more frequently during the spring months of countries having a Mediterranean type of climate. It is a rare dermatosis in Uganda, where maize is seldom eaten. It is commoner in South Africa (Gillman & Gillman, 1951), or in Italy (Frontali, 1952), where it is accompanied by hyperpigmentation of the exposed areas. The latter investigator has shown that this dermatosis responds rapidly to *small* doses (large amounts are very dangerous) of niacin and only very slowly to skim milk powder, which, however, contains small amounts of tryptophan and of the Vitamin B complex. This explains why cases at one time were considered to suffer from "infantile pellagra." It must also be admitted that transitional conditions between "enamel-paint dermatosis," pellagrous dermatosis, and "mosaic skin" are frequently seen. These three skin conditions appear to be allied in many ways. They all appear in response to some external injurious agent. They tend to be hyperpigmented. They all tend to desquamate, although to different degrees and at different rates.

It is not for me, as a clinician, to say much about the stellate fibrosis which, in Uganda, affects the portal traids and appears first in certain cases after the fatty liver of acute kwashiorkor in childhood. This is described by my colleague, Professor J. N. P. Davies, in this monograph (page 714). One thing is certain and that is that this stellate fibrosis very slowly increases during later childhood, adolescence, and adult life, while the patients show no signs of severe kwashiorlor; but, taken as a group, they continue to receive the same diets with a low protein-calorie ratio. The diets do not differ from those

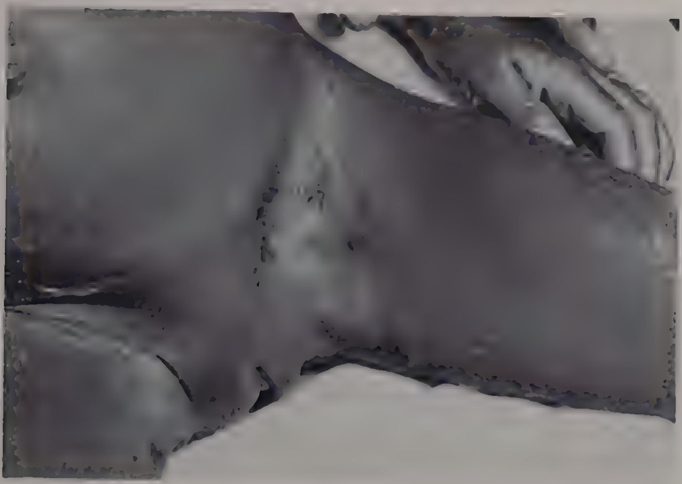

FIGURE 4

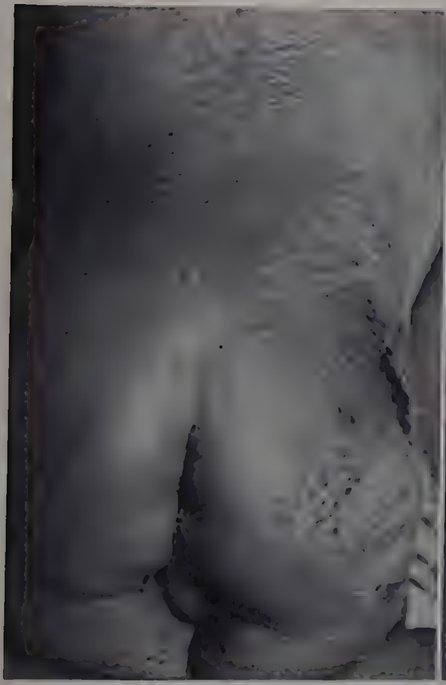

FIGURE 5

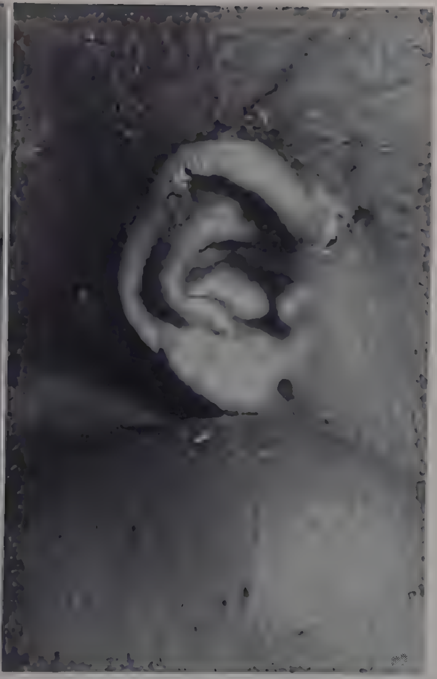

FIGURE 6

FIGURE 4 (above). African child with severe kwashiorkor, showing plaques of "enamel-paint dermatosis" in groins and left thigh; central areas have peeled to disclose pale skin; other plaques on abdominal wall have peeled to reveal punctate hypopigmentation. FIGURE 5 (below left). African child with severe kwashiorkor; large areas of a dermatosis intermediate between "enamel-paint" and "crackled-skin" are peeling over a pressure-site. FIGURE 6 (below right). African child with severe kwashiorkor and fissures in flexure of the pinna and on inner rim of auricle.

taken by infants. It is only the requirements which have altered. In my opinion, these persons often continue to show signs suggestive of mild kwashiorkor; that is to say, they are underweight and have dyspigmented hair and a slightly low serum albumin and a high gamma globulin. Frank cirrhosis of the liver, unassociated with alcoholism or manifest tropical disease, is very common in Uganda, even in young adults in the third, fourth, and fifth decades. In my opinion, progressive stellate fibrosis often leads to frank cirrhosis in Uganda, and all varieties of transitional forms are seen. Acute necrosis of the liver, in any form, is very rarely seen in children in Uganda. It occurs in adults and may lead apparently to cirrhosis. It is impossible and undesirable to do more than mention necrosis here, largely because necrosis of the liver has been reported as occurring in kwashiorkor. This is extremely doubtful, except possibly a necrosis of individual hepatic cells, and almost no reference can be traced to this in any original communication on kwashiorkor.

It is not proposed to devote any further time to a consideration of the other signs of vitamin deficiency. These are seldom seen except in the terminal states of kwashiorkor, and it should be noted in passing that signs of vitamin deficiency seldom occur in children or adults dying of general undernutrition due to calorie deficiency. Vitamin A deficiency appears to be very common in cases of kwashiorkor in Asia. Riboflavin deficiency is severe on maize diets. Both scurvy and rickets are said to occur, but are rare, especially the latter, in most areas. Thiamine deficiency has not been demonstrated in Africa, but is considered to be present in some cases in Italy (Frontali, 1952).

It will be noted that, in all this description of the clinical state, no reference has been made to the liver. In many cases of kwashiorkor, the liver does not appear to be enlarged during life; and there is little data to suggest that the degree of enlargement has been assessed in cases coming to autopsy. In Uganda, it is seldom enlarged during life or at postmortem. Abdominal distension may obscure enlargement. On the other hand, in certain parts of the world, moderate enlargement is often detected during life (Waterlow, 1948; Altmann, 1948) and I have seen cases showing enlargement in Kenya, the Sudan, Nigeria, the Gold Coast, and South Africa. If, however, the liver of any severe case of kwashiorkor, whether enlarged or not, is biopsied, marked fatty infiltration is almost always seen. In noninfected cases, this commences first, and disappears last, at the periphery of the lobule; that is, near the portal vessels. Any direct transition from the fatty liver of acute kwashiorkor into frank cirrhosis (meaning gross disorganization of liver architecture by fibrous tissue and nodules of hyperplasia) has never been recorded in any part of the world in kwashiorkor in infancy.

It is perhaps as well to close this description of the clinical picture by noting that the majority of cases of kwashiorkor in tropical regions have some infection present.

Many of these, such as the hookworms, deplete the body of proteins and increase the dietary requirements. In the majority of cases, in my opinion, it is very doubtful if these tropical infections play any significant part in producing kwashiorkor, and recent work (Welbourn, 1953) suggests that, even in the tropics, bouts of respiratory infections are the commonest diseases to precipitate an attack of severe kwashiorkor. In many cases, these infections decrease the intake of food, and of protein. Certainly, in most cases, although not in all, treating the infection without improving the diet causes almost no improvement in any of the manifestations of kwashiorkor. If any infections are severe or produce fever, it is essential to treat them while administering suitable dietetic treatment.

This clinical picture would not be complete without reference to the biochemical changes. These are regarded as constant features, and it is desirable that communications on this disease should include biochemical data. The most constant changes are a marked reduction in the serum albumin, in all the pancreatic enzymes (Thompson & Trowell, 1952) and in the plasma esterase and plasma lipase (Srinivasan & Patwardhan, 1952), in serum amylase, cholinesterase, and alkaline phosphatase. There are also a reduced blood urea, total cholesterol, and ester cholesterol (Dean, 1952b). Feeding with protein-rich diets produces a characteristic biochemical pattern of recovery, which is characteristic of kwashiorkor (Dean & Schwarz, 1953). In terms of pathology, there are changes not only in all the organs with a rapid turnover of protein (small intestine, pancreas, and liver), but all exocrine glands show changes, and there are significant changes in many organs, including the endocrine glands, especially the suprarenal (Davies, 1952).

THE TREATMENT OF CASES OF SEVERE KWASHIORKOR

The mortality rate in untreated cases with edema and anorexia is almost 100 per cent, and little improvement was effected until pathology and biochemistry had afforded some insight into the mechanisms involved. In the tropics, at one time, these cases were ascribed entirely to some tropical infection or to congenital syphilis, and treatment remained unsuccessful until the diets were also improved.

The discovery that many cases had no significant infection and the recognition of the disease in temperate regions aroused hopes that the condition might be a clinical entity and be nutritional in origin. Attention at that time, 1930–1945, was erroneously focused on the vitamins, especially those of the Vitamin B complex and on liver extracts. No serious advances were made in treatment until pathology (Davies, 1948) afforded some insight into the difficulty of digesting food, and experimental work, largely in the United States and in Europe, focused attention on the fatty liver. Although the use of known lipotrophic factors has, on the whole, proved disappointing, yet workers in the tropics felt that this disease was probably an aspect of protein malnutrition. The detection of the pancreatic lesion, of the reduction in their enzymes, and of the changes in the small intestine have all helped to explain the difficulty in digesting food.

Treatment remains difficult, although the mortality has fallen to below 10 per cent in uninfected cases. Deaths are usually due to collapse soon after admission, or very serious infections, such as a fulminant pneumonia or tuberculosis. The extreme anorexia, the difficulty in digesting food, an intolerance to all fats, and to many carbohydrates and sugars including glucose and lactose makes treatment difficult. Skim milk powder or, in the most severe cases, calcium caseinate (a more purified form of skim milk protein and one which contains little lactose), combined with a suitable form of carbohydrate, remains the treatment of choice and may initially be given by tube-feeding. The principles and methods of treatment have been set forward recently by Dean (1952a). It is important to start treatment of the infant somewhat slowly with about 20 gm. daily of protein, derived from about 60 gm. of ordinary skim milk powder. For calories nothing appears better than fully ripe bananas, which are well tolerated as in celiac disease. After a few days or weeks, depending largely on the character of the stools, the diet can be increased very slowly, until eventually children of two years are receiving about 5 gm. of protein per kg. of body weight (after loss of the edema) and over 100 calories per kg. That is to say the diet has about 5 gm. protein per 100 calories, almost all the protein, at least at first, being derived from animal sources. Dean (1952a) has reported cures with vegetable protein supplemented by vitamins, including B_{12}, but in advanced cases skim milk protein remains the most efficient form of treatment.

Within a few days, a definite response occurs. The general condition improves, mental alertness returns, appetite sharpens, and brisk diuresis occurs. The edema rapidly decreases so that the weight drops, and the "enamel-paint dermatosis" and "flexural fissures" start to heal. The stools improve but tend to remain bulky and contain undigested food, and diarrhea is apt to recur if much carbohydrate or fat is taken and after every successive addition to the diet. The pancreatic enzymes increase, the serum albumin rises very quickly, the gamma globulins increase and tend to remain high for a very long period of time (Anderson & Altmann, 1951).

As assessed by serial liver biopsy, fat starts to disappear rather slowly from the center of the hepatic lobules, that is around the central vein, but even after 4 to 8 weeks of treatment, a certain amount of fat is often still visible at the periphery of the lobule. Milk, meat, and whole liver and crude liver extracts all exert a lipotrophic action. It is difficult in histological studies to say whether any of these foods were more effective than another (Trowell, 1949). It is doubtful if special supplements of methionine or choline have been demonstrated to accelerate this slow lipotrophic action of whole animal protein; although, in Chile, Meneghello and Niemeyer (1950) considered that some acceleration occurred in infants given 5 gm. of choline hydrochloride daily, in addition to 3.5 gm. milk protein per kg. of body weight.

It is but natural that it should be desired to study the effect of other nutriments, such as individual amino acids and vitamin B_{12} but this is very difficult, if not dangerous, in severe cases of kwashiorkor for, unless they receive skim milk protein, they soon die. It is, therefore, essential to take cases that are less critically ill, prescribe a diet which will prevent further deterioration, but which will maintain them in almost a stationary condition during a test period of 10 to 20 days, and then to add the single nutriment, the effect of which it is desired to study. In actual practice, this is difficult and dangerous. Observations so far have not demonstrated that supplements of methionine aid recovery. No other amino acid has been tried by more than one observer. No clinical response to vitamin B_{12} has been unequivocably demonstrated.

The administration of this vitamin, even parenterally, does not improve the anemia, which is usually normocytic but may be slightly macrocytic. The bone marrow is probably very seldom megaloblastic as in pernicious anemia. It is usually normoblastic or macronormoblastic. The anemia does not respond to folic acid or to refined liver extracts, but it sometimes improves slowly while receiving iron. Anemia is seldom severe unless some infection is also present. It is probable that the anemia is due to protein deficiency and resembles that present in cases of massive albuminuria.

A marked feature of many cases of severe kwashiorkor is that progress is almost always slow and many months often elapse before the child is normal in every respect. No reason is known which will explain the very slow gain of weight usually encountered during the later months of treatment. In this respect, the recovery of cases of kwashiorkor appears fundamentally different from that seen in children of the same age who are recovering from a period of

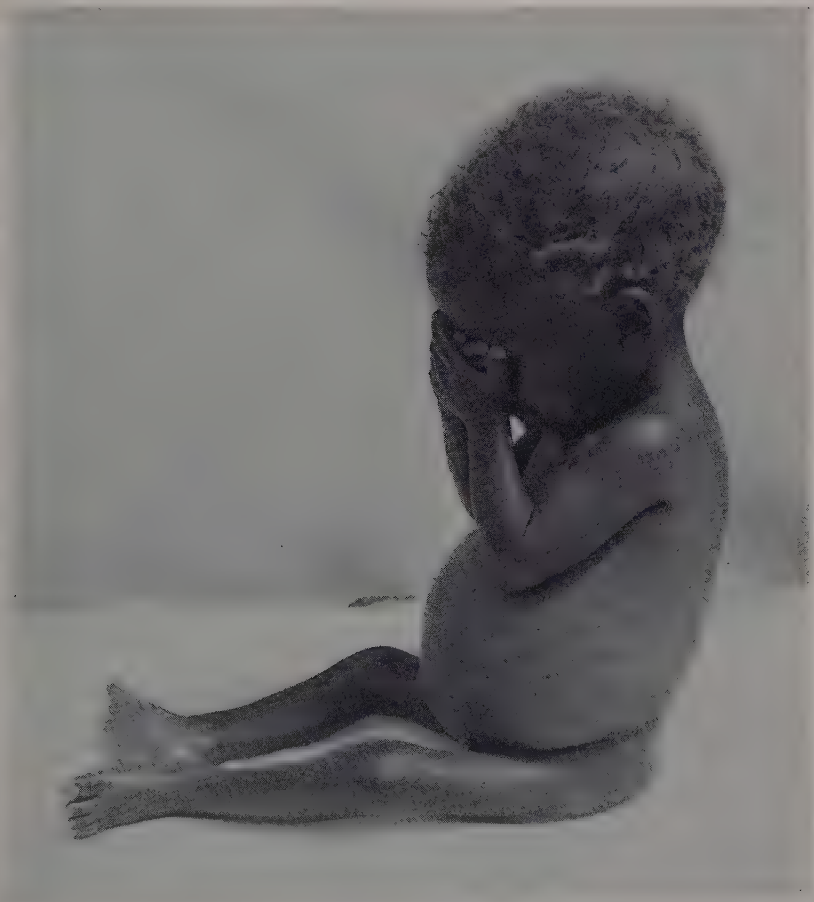

FIGURE 7. Convalescent kwashiorkor, showing pot belly, thin limbs, and pale hair.

undernutrition and calorie deficiency. Appetite is apt to remain indifferent and diarrhea is easily provoked in all cases of kwashiorkor. The failure to gain weight rapidly has not so far been explained by any infection, or any irreversible change in the pathology or biochemistry. Probably more attention should be paid to the intake, the digestion and utilization of food, since it is probable that the first organ to suffer and possibly the last to recover is the gastrointestinal tract. The pancreas and liver are possibly affected later and this sequence reflects the high protein requirements of these organs. Clinically, these convalescent children have swollen abdomens and thin limbs (FIGURE 7) as two American doctors noted (Payne & Payne, 1927) when they described kwashiorkor in the New World, but unfortunately for them, they suggested no satisfactory title and called the complaint "Edema disease of Haiti."

In conclusion, I should like to state that, if I were asked what clinical signs are of the greatest significance in kwashiorkor, I would answer, without hesitation, "Appetite and interest." In the early decrease of appetite, which is almost abolished in all cases of severe kwashiorkor, this disease differs fundamentally from undernutrition on diets, normal in the proportion of the various nutriments, but reduced in the total amount. In kwashiorkor, there is little desire for food and little interest in life. These are the stigmata of kwashiorkor. Is this a response to the fatty liver or, as I suspect, due to some change in the functions of the hypothalamus, together with its numerous nervous connections and subject to various forms of chemical control? Why should appetite decrease possibly at an early stage on diets having a low protein-calorie ratio? How does skim milk powder stimulate hunger in a child dying of kwashiorkor? Eventually, it stimulates interest in life and, when a child with kwashiorkor smiles, he is well on the way to recovery.

Summary

In recent years a disease-process has been described in young infants. It is common in tropical countries and whenever the diet of a young child contains a low protein-calorie ratio especially if the proportion of animal protein is low and that of carbohydrate is high. The disease is considered to be identical to Mehlnährschaden and many cases have been described as "nutritional edema." The latter term describes two or more disease processes: firstly, undernutrition terminating as hunger edema, in which the diet, although balanced in its proportions is small in amount and deficient mainly in calories; and secondly, kwashiorkor produced usually by a low-protein, high-carbohydrate diet, fed in amount adequate to satisfy appetite. Only in the latter disease does much fat accumulate at the periphery of the hepatic lobule and then spread to other parts of the lobule. The liver may enlarge, but often remains normal in size. Kwashiorkor has a characteristic pathology and biochemistry and pattern of recovery.

The clinical picture of cases of severe kwashiorkor in childhood is described. This is very characteristic although, as terminal states, various signs of vitamin deficiency may appear. At an early stage in the illness, it is probable that those organs which have the highest protein turnover are affected, with selective

atrophic changes and loss of secretions by the small intestine, pancreas, and fatty changes in the liver.

These features all respond to protein-rich diets, especially milk protein and animal protein, and it is doubtful if any specific response has been demonstrated to methionine, choline, vitamin B_{12}, or folic acid. There is uncertainty concerning the mechanisms and the factors which are involved. Animal protein, especially milk, is still the best treatment of advanced cases, but prevention and the cure of the more numerous mild cases must lie in an increased intake of vegetable protein, fortified possibly by factors unidentified at present.

Apathy and anorexia are the stigmata of kwashiorkor and separate this disease-process from that seen in undernutrition on a diet, normal in its proportions, but reduced in its amount, thus producing a disease-process in which calorie deficiency dominates the response of the body.

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VITAMIN E AND CAROTENOIDS IN THE BLOOD PLASMA IN KWASHIORKOR

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Deficiency of protein has been generally accepted as one of the main factors in the development of kwashiorkor. Other dietary deficiencies which might aggravate the want of protein therefore deserve attention. Experiments with animals have clearly demonstrated that their ability to subsist on diets low in protein is influenced by the allowance of vitamin E. Thus Dam[1] found that young rats which were given a diet grossly deficient in protein survived longer when they were dosed with vitamin E than when they were left undosed. Various other workers confirmed the beneficial effects of vitamin E in rats given diets low in protein both upon the body weight and in preventing dental abnormalities.[2, 3, 4, 5] In view of the severe injury to the liver in kwashiorkor, special interest may be attached to the finding of Schwarz[6] and others [7, 8] that vitamin E protects rats against the hepatic necrosis which is otherwise induced by protein deficiency.

It seemed desirable, therefore, to try to obtain evidence on the vitamin E status of patients with kwashiorkor by estimations made on the blood plasma. Specimens were sent by air from Uganda to Cambridge, mostly in 1950 but a few in 1951. The plasma was shaken up with ethanol and light petroleum, and total carotenoids were estimated by their yellow color in the extract separated after centrifuging. The extract was then evaporated, and the residue was redissolved in hot ethanol. Cholesterol and other sparingly soluble substances were removed by freezing and again centrifuging and "total reducing substances" were estimated in the supernatant solution with $\alpha\alpha'$-dipyridyl and ferric chloride. "vitamin E" was measured as the difference between the total reducing substances and the correction appropriate for the carotenoids. For purposes of comparison, estimations were also made upon specimens of plasma from healthy male African adults and from a few healthy infants. Specimens were also taken from adults suffering from pyomyositis, which is a disease characterized by degeneration of the voluntary muscles and the formation of abscesses.

From TABLE 1 it will be seen that lower levels were found for vitamin E in kwashiorkor (average 0.67 mg. per 100 ml.) than in healthy men (average 0.92 mg.) or in four healthy infants (average 1.10 mg.). The reduction of carotenoids in kwashiorkor, with an average value of 23 μg. per 100 ml. as compared with 99 μg. in men and 107 μg. in healthy infants was even more noticeable. Several of the specimens of plasma gave extracts which appeared quite colorless to the eye. The levels in pyomyositis also fell below the values for healthy subjects both in vitamin E and carotenoids.

From these results it would appear either that the intake of vitamin E and carotenoids is low in kwashiorkor or that the disease prevents their efficient absorption from the intestines or carriage by the blood plasma. Evidence of

TABLE 1

	No. of cases	Av. age	Carotenoids µg. 100 ml.		Tocopherol mgm. 1000 ml.	
			Range	Av.	Range	Av.
Kwashiorkor infants..........	12	$2\frac{6}{12}$	4– 54	23	0.35–1.06	0.67
Normal African infants.......	4	$2\frac{3}{12}$	46–142	107	0.85–1.40	1.10
Normal African adult males...	10	A	49–167	99	0.58–1.37	0.92
Pyomyositis in adults........	17	A	13– 89	38	0.34–1.38	0.80

low values for the vitamin E has already been found by Darby *et al.*[9] in patients with absorptive defects; the range was 0.1–1.5 mg., and the average 0.8 mg. as compared with 0.5–1.5 mgm., average 1.0 mg. in healthy adults. Filer *et al.*[10] found very low levels in infants and children suffering from fibrocystic disease, cirrhosis of the liver, sprue, celiac disease, and diarrhea. All patients with these diseases had their lowest readings in repeated observations within the range of 0.06–0.25 mgm. The values found in kwashiorkor, therefore, may seem no lower than might be expected in a disease which is characterized by damage to the liver and general impairment of the absorption of food. It is perhaps surprising that the ranges observed did not differ more widely from those found in pyomyositis, a disease in which injuries to the liver are less prominent than in kwashiorkor, but in which a suspicion of vitamin E deficiency might possibly be suspected from the muscular degeneration. Presumably the frequency of fever influenced the levels in this disease.

Even if we regard the reductions in vitamin E and carotene mainly as secondary effects of kwashiorkor, however, they must not be dismissed too lightly as having no part in the development of the disease. Evidence of ceroid pigmentation suggestive of secondary deficiency of vitamin E deficiency has been reported by Pappenheimer and Victor[11] in subjects who have died after obscure diseases involving chronic diarrhea. Tverdy *et al.*[12] have made similar observations in a case of sprue. It cannot be ruled out, therefore, that a secondary deficiency of vitamin E may be included among the numerous complications and aggravations which are involved in the progress of kwashiorkor. Our very low values for carotene suggest further that the vitamin A status may be endangered when the reserves of the preformed vitamin which are presumably stored during lactation have been used up.

Summary

Low values for vitamin E and carotenoids, probably related to impaired powers of absorption, were found in the blood plasma of African infants suffering from kwashiorkor.

The possibility of the occurrence of secondary deficiencies of subnormalities of vitamins E and A as complications to kwashiorkor should not be overlooked.

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ASPECTS OF NUTRITIONAL LIVER DISEASE—HUMAN AND EXPERIMENTAL

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In this communication we propose, first to examine the relationships of hepatic fibrosis to antecedent fatty change and to necrosis of the liver in malnourished human beings and in animals fed various diets and, secondly, to consider methodological problems of nutritional research, particularly as they affect the assessment of the role of specific food factors in promoting or preventing liver disease.

The Fatty Liver and Hepatic Fibrosis

A fatty change can be found in livers of malnourished infants and children admitted to hospital during an acute nutritional breakdown (Normet, 1937; Bablet and Normet, 1937; Gillman *et al.*, 1945, 1951; Waterlow, 1948; Van der Sar, 1951; Oomen, 1951; Altmann, 1948). By serial biopsy examination, we have demonstrated (a) that, under appropriate therapy, large accumulations of fat may disappear from the liver of an infant pellagrin within a period of fourteen days; (b) that a mildly fatty liver in an infant pellagrin admitted to hospital can become intensely fatty within a period of eight days; and (c) that a malnourished infant or child recovering from an acute episode may leave hospital with only a small residue of fat in the liver.

Six-monthly biopsies of the livers of infants and children who have left hospital after recovery from an acute nutritional breakdown show no evidence of persistent fat in the liver even though such infants and children, through necessity, continue to consume inadequate diets.

From a survey of 262 livers of native (African) infants and children dying, from all causes, between the ages of birth and eight years, and 82 livers of infant and child pellagrins admitted to hospital, we arrived at the conclusion that although fatty livers may be associated with many diseases, fatty change is more frequent and is likely to be more severe in African infants admitted to hospital with nutritional edema or infantile pellagra than in children of the general population.

The fatty liver in infants suffering from nutritional edema and pellagra is therefore of relatively acute onset and in a sense is similar to the intensely fatty livers observed in well-nourished children dying four to eight days after an acute attack of "summer diarrhea" or after severe burns. Like the other events associated with the acute episode, a fatty liver is an expression of a relatively sudden and profound disorder of metabolism occurring in a malnourished infant (Gillman and Gillman, 1951).

One question now arises: whether the fatty liver in any way influences the development of fibrosis. At the outset, we may say that portal fibrosis, or more than usual amounts of fibrous tissue, in the liver occur frequently in adult Africans (Gillman and Gillman, 1951). It now remains to attempt a correlation between the frequency of hepatic fibrosis and steatosis.

Twenty-five of 261 African adults (*i.e.*, 9.5 per cent) dying accidentally showed gross portal fibrosis as compared with 11.9 per cent of 177 adult African pellagrins admitted to hospital. In the general population, the incidence of the fatty liver in Africans dying from accidental causes was 2.7 per cent.

In the livers of 79 adult females over the age of twenty years admitted to hospital during an attack of pellagra, 66 (86 per cent) contained some stainable fat and, of these, the livers were moderately or intensely fatty in 35 females (46 per cent). On the other hand, some fat was detected in the livers of 65 (69.1 per cent) of 94 adult male pellagrins in our series and, of these livers, 33 (35 per cent) contained moderate or abundant amounts of fat. Apart from demonstrating the greater incidence of fatty change in females, this analysis reveals that a fatty liver occurs much more frequently in pellagrins than in members of the general population.

We are led to conclude that there is no *prima facie* case for claiming the existence of a direct causal relation between the fatty liver and hepatic fibrosis in the malnourished African. The evidence adduced in this connection is as follows: (1) The fatty change in the livers of infant and adult Africans is of relatively acute onset; (2) After the acute episode the fat usually disappears from the liver; (3) Sampling of the livers of the general population at all ages affords no evidence of a persistent and progressive fatty change; (4) The evidence of hepatic fibrosis in the general population of Africans cannot be correlated positively with the frequency of hepatic steatosis. Taking this evidence into account, we affirm once more that the fatty liver, *per se*, in the malnourished infant and adult African suffering an acute nutritional breakdown plays no direct role in promoting the high incidence of liver disease in Johannesburg. Subsequent to our own study, Dible (1951) independently arrived at the conclusion that, even in London, there was little evidence to show that fatty infiltration of the liver was a precursor of hepatic fibrosis. But it has been shown by Dible (1951) and by Gillman and Gillman (1951) that a fibrotic liver can undergo a fatty change, in which case it might easily be possible to mistake the sequence of these two reactions and to regard the fatty change as preceding the fibrosis.

The frequent coexistence of fat and fibrosis in the same liver has led to the generally held opinion that long-standing fatty change in the livers of animals on various diets is an essential precursor of diffuse, hepatic fibrosis (Himsworth, 1947). This view implies that the fat-filled, enlarged hepatic cell occludes the circulation through the lobule and eventually results in necrosis of the parenchyma. Necrosis, in turn, is said to stimulate the connective tissue reaction, an essential precursor of hepatic fibrosis. It would thus appear that, in the fatty liver, as in the necrotic liver to be mentioned below, necrosis is the precursor of hepatic fibrosis.

From our own observations in animals we have demonstrated that certain diets will favor the development of an extensive fatty change which can persist for approximately 300 days without any evidence of fibrosis (Gillman *et al.*, 1953). Since it is possible to disengage the fatty change from fibrosis, it follows that the accumulation of fat and the overgrowth of fibrous tissue in the liver are end results of two separate processes which are not necessarily causally related to each other. Fibrosis can be disengaged from steatosis. Each reaction can occur independently of the other, and each requires special conditions if the appropriate reaction is to be set into operation. Nutritional experiments in animals have shown that, as a rule, fibrosis only supervenes in a fatty liver if the latter is progressive and long-standing. Such a relationship, we have shown, does not exist in malnourished Africans. Although nutrition plays a commanding role in promoting the frequency of fibrosis in the malnourished African, it does so in other ways than by exciting an antecedent intense, fatty reaction.

We wish to point out that the fatty change in the liver may participate indirectly in the development of hepatic fibrosis. An intensely fatty liver in pellagrins is indicative of an acute metabolic disorder. On each occasion when the liver undergoes a fatty change, there is a katabolic process of greater or lesser severity which has, as one of its consequences, irritation of the reticulo-endothelial system. Repeated irritation of the reticulo-endothelial system over many years, often beginning in infancy, can, in some circumstances, culminate in portal fibrosis.

Necrosis and Hepatic Fibrosis

Acute necrosis of the liver has not been established in infant and adult Africans admitted during an acute nutritional breakdown. It is true that puncture biopsy only provides a fragment of liver tissue. Consequently, we may have missed many cases of liver necrosis. This possibility is unlikely, since we have found no evidence of necrosis in any of the livers of our cases coming to autopsy. We would like to mention that the administration of thiamin to one of our pellagrins unquestionably precipitated diffuse necrosis of the liver, recognized from fragments obtained by puncture biopsy (Gillman and Gillman, 1951).

There are other circumstances, however, usually those associated with acute anoxia, which may contribute to necrosis of the liver (Gillman and Gillman, 1948). In the first place, the frequent use of open braziers to heat ill-ventilated huts and shacks during the winter months leads frequently to carbon monoxide poisoning. In a small series of 25 cases of fatal carbon monoxide poisoning, we observed hepatic necrosis in two cases surviving for longer than nine hours. Secondly, severe hemorrhage, resulting from industrial or other accidents and from assaults during drunken brawls, produces severe anoxic reactions in the liver. If such individuals survive for a few days, the anoxic reaction may eventually culminate in necrosis. We reported two cases of extensive necrosis of the liver following severe hemorrhage in forty cases of multiple injuries. It must be remembered that many of the cases died within a few hours of the injury, but they did show an intense reaction in the form of vacuolated and plantlike cells which, as we have shown, can go on to necrosis. Similar plantlike cells apparently precede the necrosis of infective hepatitis, as shown experimentaly by Kyo Hara *et al.* (1952).

Thirdly, severe infections, especially those associated with lobar and broncho-pneumonia, often precipitate jaundice in the African. In some of these cases coming to autopsy, we have found necrosis of the liver. Fourthly, prior to the introduction of antibiotics, intensive and often indiscriminate use of arsenic, ostensibly for the treatment of syphilis among the Africans, may also contribute to the production of hepatic necrosis. We mention this because of the numerous severe reactions we have observed in Africans receiving arsenic, who were sufficiently indisposed to be admitted to hospital. But not all patients, even though indisposed, seek hospital treatment after arsenic therapy. The untoward reactions experienced are often regarded as part of the treatment. Fifthly, the frequency of tuberculosis among the Africans in Johannesburg and elsewhere in this country is so great that it is regarded as a major health problem. Extensive tuberculosis of the liver is a common finding. Even when less extensive, we have found more than suggestive indications that tuberculosis can contribute to a low grade but widespread destruction of liver tissue.

In this connection, we should like to mention that Straub and Schaberg (1950) have drawn attention to the frequency of hepatitis, allegedly due to a virus, in malnourished Indonesians. Perusal of their protocols disclosed that, in every instance cited, a large or small focus of tuberculosis in the lung was noted. Straub and Schaberg regard the prevalence of hepatic fibrosis in Indonesia as an interaction of two factors; namely, malnutrition and virus infection. While we are not entirely satisfied that Straub and Schaberg have made out a case for virus infection in the Indonesian, nevertheless, in view of the greater vulnerability of the liver to infection in malnourished individuals, it seems to us that the role of the tubercle bacillus, as an etiological agent in the production of hepatic necrosis and fibrosis in malnourished individuals, has not been adequately assessed. Moreover, it is our opinion that not only virus infection and tuberculosis but also parasitic infections, such as schistosomiasis and malaria, as well as other infections, including those responsible for lobar and broncho-pneumonia, may contribute collectively, either directly by destruction of liver tissue, or indirectly through the production of severe anoxia, to the greater incidence of necrosis of the liver in a chronically malnourished population.

Although we have seen cases with acute and subacute yellow atrophy of the liver in Africans, it is impossible, in the absence of further laboratory examinations, to incriminate the virus of infective hepatitis. Besides, the use of medicinal herbs by Africans has been repeatedly suspected of promoting extensive necrosis of the liver of the type mentioned above.

As in the case of the fatty change, necrosis has been regarded as a precursor of hepatic fibrosis. However, it is important to realize that fibrosis is not an automatic response of the liver to necrosis. Necrosis can be disengaged from fibrosis. Both in man and in animals it has been repeatedly demonstrated that, even after extensive injury to the liver, regeneration may be complete [Dible *et al.* (1943); Lucké (1944); Cameron and Karunaratne (1936); Orr (1940); Gillman *et al.* (1952 b)]. On the other hand, we have demonstrated in animals that whether or not hepatic fibrosis will supervene in a liver which has undergone extensive necrosis of dietary origin is not only a function of necrosis but also of the diet. If, after necrosis has declared itself, the animals are still maintained on the deficient diet, recurrent attacks of necrosis are to be expected. In this context, provided the animals survive for any length of time, hepatic fibrosis and even nodular hyperplasia are almost inevitable consequences. On the other hand, if necrosis has declared itself and the animals are then given access to a balanced ration, regeneration of the liver is usually complete and fibrosis is prevented. Moreover, by allowing the animals access alternately, for varying periods, to the necrogenic and to the balanced diet, it is possible to produce a variety of pathological reactions in the liver. These can be interpreted as representing a combination of necrosis, regeneration, and fibrosis. Gross distortion of liver structure can be one of the end pictures (Gillman *et al.* 1952, a and b).

It is evident from the latter experiment that, although survival of animals fed a necrogenic diet can be prolonged by occasional feeding, for limited periods, of a balanced ration, the liver still remains vulnerable and can still be grossly disorganised on a later occasion. Clearly, the more chronic forms of liver disease can be attributed to the prolonged subsistence of animals on a bad diet, punctuated by short exposure to the effects of a good diet. Noteworthy, too, was the great individual variation in the way the liver reacted to this dietary regimen. The individual difference in reactivity may be attributed, in part, to differences in the ability of rats to take advantage of the good diet, which is, in turn, determined by the extent of pre-existing liver and other organ damage imposed by the necrogenic diet.

Furthermore, it is apparent from these animal experiments that the mechanism responsible for necrosis is not identical with that favoring fibrosis. Both phenomena required special, and perhaps different, metabolic conditions for their emergence. Whenever necrosis has supervened, it has been attended by fibroblastic activity only when the prevailing metabolism is appropriate. If necrosis has declared itself and if the animal is then given access to a balanced diet, restitution of liver structure and not fibrosis will be the end result. Apart from the greater vulnerability of the liver of malnourished individuals to necrosis, it is significant for the understanding of hepatic disease in man that both necrosis and fibrosis can be induced in the liver by diet. The animal experiments indicate that, if necrosis of the liver is precipitated by deficient diets, the supervening fibrosis is to be regarded as a natural consequence in contrast to regeneration in better fed animals. Diet then may serve to increase the frequency of hepatic fibrosis in two ways: firstly, by rendering the liver more vulnerable to noxious agents and especially to the effects of acute anoxia; and, secondly, by promoting fibrosis once necrosis has supervened.

One question still remains unanswered: Why is there such variability in liver pathology in different members of the same malnourished population? The answer may be given, in part, in terms of the subtle differences in diet consumed by members of the population; the extent to which each individual may gain access to better food during times of plenty; the presence or absence of various infections, including pneumonia, tuberculosis, malaria, schistosomiasis, *etc.*; and the extent to which occupation and living conditions expose individuals to severe liver injury.

In addition, the difference in the reactivity of livers of malnourished individuals probably has the same basis as the differences observed in the livers of seemingly well-nourished patients contracting infective hepatitis during the same epidemic. Of those suffering from infective hepatitis, some may suffer a mild attack and recover rapidly, others develop hepatic necrosis which heals completely, whereas in still others the necrosis may go on to fibrosis. In these instances, the virus of infective hepatitis merely served to expose underlying differences in metabolism of affected individuals already existing even before the disease was contracted. That is to say, although cases contracting infective hepatitis but reacting differently to the virus had ready access to a good diet and were not showing signs of malnutrition, such individuals were probably utilizing their food in different ways. After all, it is well known that two individuals, consuming similar diets, may put the nutrients to different uses. One individual may increase rapidly in weight and another may remain thin.

In this connection, we may recall the classical experiments of Chaikoff and his associates (Chaikoff *et al.* 1938, 1948; Gillman and Chaikoff, 1949) who demonstrated the altered utilization of food in thyroidectomized, hypophysectomized and pancreatectomized dogs. Fatty livers developed in all these animals while consuming a diet rich in lipotropes and adequate to prevent a fatty liver when such diet was fed to normal dogs. However, the fatty liver of the dogs with one or other of the endocrine glands ablated, could be prevented by increasing the choline intake in amounts which ordinarily would not be available in an average diet.

By analogy, human subjects suffering from varying degrees of subclinical hypothyroidism or of pancreatic disease will not utilize the same food in the same way as another individual with a normally functioning thyroid or pancreas. Indeed, as revealed in the case of hypothyroidism, nutritional disease may even supervene on a diet adequate to maintain an intact organism in good health (Gillman and Gillman, 1951). If then, two individuals, one in good health, the other suffering from subclinical hypothyroidism, are both consuming the same diet, the diet will, in the one case, be adequate and, in the other, deficient. If these two individuals both develop an attack of infective hepatitis, divergences in hepatic reactions are to be expected in the same way as has been shown in the case of the reaction of livers of animals subsisting on different diets and poisoned with the same concentration of chloroform. The divergent reaction of livers to the same stimuli, therefore, is dependent on the prevailing metabolism. The latter is not only a function of the diet consumed, but also of the way the nutrients are utilized. The utilization of food in turn is dependent on the functional integrity of the various organs and tissues and the way these various components of the organism are functionally related (Gillman and Gillman, 1951). The difference in liver structure observed in a random sample of a malnourished population, therefore, can be explained on the same basis as mentioned above in the case of infective hepatitis.

In addition, however, consideration needs to be given to the number of previous nutritional breakdowns, the age at which the first breakdown occurred, the extent of collateral organ damage precipitated by infections, *etc.*, as well as to the particular economic circumstances of each individual from birth until the time the liver is examined.

The search for an understanding of the mechanism underlying the prevalence of hepatic disease and more especially of hepatic fibrosis in a malnourished population demands a wider appreciation of the complex factors involved in a biological reaction. As will be indicated in the final section of this paper, the approach to the problem of nutritional disease is not to be sought only in terms of single factors.

Methodological Considerations of Nutritional Investigations

It has become common practice among experimentalists to design diets in such a way that the presence or absence of a specific factor may decide whether or not spontaneous liver disease will emerge, or whether or not the liver will be protected against toxic agents. As a consequence, they have identified many substances which, in particular dietary contexts, play a part in preventing or promoting liver disease. Thus, it is now well recognized that, by changing the context of the diet, a particular food factor may no longer protect the liver against spontaneous "injury."

With the exception of cystine, the general trend has been toward accepting liver disease as the consequence of a lack of one or other constituents in the diet. The inclusion of 1 per cent cystine in the diet can lead to extensive hepatic necrosis and hemorrhage within a few days (Earle and Victor, 1941). In this instance, a causal relationship can be established between the contents of the diet and the onset of liver necrosis. Even in those diets where the absence of a particular factor can be shown to be related to a particular pattern of liver reaction, too little consideration has been given to defining the type of metabolism promoted by the diet which in the first instance allowed the particular factor to be all important in determining whether or not the integrity of the liver would be maintained. If the emphasis is placed mainly on the factors lacking from the diet and not on the constituents of the diet, then the problem of liver necrosis assumes bewildering proportions.

A perusal of the literature reveals that liver necrosis may be produced by so-called low-protein diets and prevented in several different ways: (1) Necrosis develops in rats fed a diet low in protein and can be prevented by the addition of choline and cystine (Glynn *et al.*, 1945), or alpha-tocopherol (Himsworth and Lindan, 1949; György *et al.*, 1950), or by antibiotics (György, 1951); (2) Necrosis of the liver in rats fed on the low protein diet used by Hove *et al.* (1949) may be prevented by tocopherol but not by methionine; (3) Necrosis of the liver in rats fed potato starch and food yeast (low in proteins) may be prevented by alpha-tocopherol or methionine, but not by cystine (Gillman *et al.*, 1952a); (4) Diffuse necrosis can occur even on high protein diets lacking vitamin A and fat, but containing alpha-tocopherol, and can be prevented and cured by the addition of vitamin A (Gillman *et al.* 1952a).

It is quite clear that, in these different circumstances, the constituents of the diet rather than the intrinsic "needs" of the organism have been collectively responsible for imposing a metabolism which required the further inclusion of a specific factor in the diet for the prevention of liver disease. Moreover, we have demonstrated that in so-called low-protein diets, factors other than protein can decrease the incidence of hepatic necrosis. Himsworth (1947) wrote: "The most discordant results were received and it is no exaggeration to say that at one time or another every dietary component save carbohydrate has been indicted (as the cause of liver injury)." We have since shown that even the nature of the carbohydrate can modify the incidence of necrosis (Gillman *et al.*, 1952a).

Clearly then, the constituents of the diet play a commanding role in creating the conditions for making a specific nutrient a limiting factor in a nutritional syndrome. If the significance of a particular nutrient is to be assessed, it is equally important that the conditions be established which allow the nutrient to occupy such a commanding role in preventing or promoting a disease process of nutritional origin. In support of this opinion, we should like to present some of our own observations on the prevention or the facilitation of the fatty liver in rats.

Using maize as the main constituent of the diet, a fatty liver is much more easily produced in rats than is hepatic necrosis (TABLE 1). If maize alone is fed to adult rats (TABLE 1, Diet 1), in the absence of any intercurrent infection or interference with food intake consequent on injury to the teeth, a fatty change, commencing as early as the sixteenth day of the experiment, declares itself in every animal, becoming evident first in the right lateral lobes of the liver. The fat increases progressively to involve the other lobes and every cell may be filled with large globules of fat. In young rats (30 gm. to 40 gm.) fed maize alone, the fatty liver does not develop (TABLE 1, Diet 2). By the addition of other nutrients, however, it is possible even in young rats to promote consistently a fatty change in the liver (TABLE 1, Diets 7, 8, 10, and 11). The most intensely fatty livers were found in young animals fed Diets 10 and 11 and the least intensely fatty, in rats fed Diet 7. If the salt mixture is omitted (Diet 9), the fatty change does not appear in the liver. Apart from congestion, no other gross or microscopically demonstrable pathology is evident.

It might be argued, according to the views expressed by Handler and Dubin (1946), that the absence of the fatty liver in young rats is due to the failure of these animals to grow to any extent. We have observed fatty livers in rats fed 10 per cent brewers' yeast containing an equivalent of 0.5 per cent added cystine and potato starch even though the growth was scarcely different from that of young rats which failed to develop fatty livers while subsisting on maize alone. Then, too, we have produced a fatty change in the livers of young rats on a diet consisting of 10 per cent vitamin-free casein, 30 per cent corn oil, 50 per cent sucrose, 4 per cent agar, 4 per cent salt mixture, and 2 per cent cystine, together with an appropriate vitamin mixture. The growth of these animals was of the same order as that of rats fed Diet 9 in which the salt mixture was absent. In the latter animals (that is, those on Diet 9) neither the fatty liver nor hepatic necrosis was observed. Clearly then, neither the fatty change nor necrosis of the liver is necessarily a function of a minimum amount of growth in the conditions of our experiment.

The quality and quantity of proteins in the diet are generally believed to

TABLE 1

Diet no:	Maize	Glucose	Food yeast	Casein	Skimmed milk powder	Salts	Fat	Vitamins A, E, & D.	Protein-content of diet & (per cent)†	No. of rats.	Average initial weight (gm.)	Average maximum weight (gm.)	Period of survival (days)	State of the liver
1	100	—	—	—	—	—	—	—	8.1	30	261	244	350–400	Intensely fatty.
2	100	—	—	—	—	—	—	—	8.1	15	34	40	65–100	Atrophic.
3	90	—	10	—	—	—	—	—	12.2	5	40	64	58– 69	Atrophic.
4	95	—	—	—	5	—	—	—	9.1	5	34	85	142–231	No fatty change. 1 focal necrosis.
5	80	—	—	20	—	—	—	—	26.5	5	35	68	62	Congested.
6	97	—	—	—	—	3	—	—	7.8	5	32	75	104–163	Mildly fatty.
7	72	—	10	5	—	3	10	—	15.7	5	40	340	184* 391‡	Mildly fatty.
8	82	—	10	5	—	3	—	+	16.5	5	28	315	244*	All moderately fatty.
9	75	—	10	5	—	—	10	+	15.9	5	28	88	65–125	Congested.
10	77	—	10	—	—	3	10	+	11.1	5	34	254	238*	Intensely fatty.
11	82	—	—	5	—	3	10	+	11.6	5	32	206	244*	Intensely fatty.
12	82	—	—	—	5	3	10	+	8.2	5	30	206	255*	Moderately fatty.
13	67	—	—	—	20	3	10	+	11.6	5	31	373	242*	No obvious pathology.
14	—	67	—	—	20	3	10	+	6.2	5	28	121	255*	No obvious pathology.
15	57	—	10	20	—	3	10	+	29.5	5	31	325	234*	No obvious pathology.
16	72	—	10	—	5	3	10	+	12.3	5	39	305	254*	4 intensely fatty.

* Represents the day on which all rats in the experiment were sacrificed.

† Protein determined as nitrogen.

‡ Rats still alive, liver biopsy at 391 days.

determine whether or not fat will accumulate in the livers of experimental animals. The numerous reports published during the last decade afford ample support for this belief. Without wishing to reopen the entire problem of the relation of proteins to the fatty liver, we should like to mention at this stage that our own investigations reveal the unsettled role of proteins in promoting or preventing the fatty liver.

From TABLE 1, it is evident that the fatty liver develops in young rats fed Diet 7 and Diet 11. In heavy-weight (adult) rats, maize alone is efficient as a fatty-liver-producing diet. If we examine the protein content of all these diets (TABLE 1), it is clear that in our experiments the fatty liver was produced with protein concentrations ranging from 8.1 per cent to 15 per cent. In the case of the maize alone (Diet 1), the fatty liver could be prevented or cured by the administration of 50 mg. of methionine per rat per day. This latter observation would seem to indicate that, even in diets with over 15 per cent protein, there is still a lack of methionine or of proteins containing an equivalent of that amino acid. Further confirmation would seem to be available from the fact that, by increasing the casein from 5 per cent to 20 per cent so that the total amounts of proteins reached 29 per cent (Diet 15), the fatty liver was prevented.

When, however, maize and 5 per cent dried skimmed milk were fed to rats (yielding an over-all protein content of 9.1 per cent), the liver did not show any fatty changes (TABLE 1, Diet 4). Similarly, when a diet containing glucose and 20 per cent skimmed milk powder (TABLE 1, Diet 14), yielding a total of 6.20 per cent protein was fed to young rats, the fatty liver was again prevented. It might be argued that 6.2 per cent of dried skimmed milk protein provided sufficient methionine to prevent the fatty liver. On the other hand, 5 per cent dried skimmed milk in the maize diet (Diet 16) contains about one third the protein in 5 per cent casein. The latter diet favored the accumulation of fat and the former did not. Five per cent casein contains more methionine than does 5 per cent dried skimmed milk. Yet the dried skimmed milk is more effective in preventing the fatty liver induced by a predominantly maize diet. It should not be forgotten, however, that, whereas casein is a pure protein, dried skimmed milk contains, in addition to some proteins, other factors which constitute approximately 69 per cent of the dried skimmed milk. These other factors were apparently as important as the small amount of protein in preventing hepatic damage. It is imperative to stress the fact that dried skimmed milk, or even whole milk, cannot be regarded as consisting only of proteins, however efficient dried skimmed milk may be in treating malnourished infants in Uganda [Dean, 1952; Trowell *et al.* (1952); Editorial (British Med. J., 1952)]. Since we have already indicated that the omission of salts from a maize diet can prevent the fatty liver (compare Diets 9 and 10), it follows that explanations of the complex chemical processes underlying the production of the fatty liver in terms of single factors are untenable.

From these seemingly paradoxical observations, it is evident that, as in the case of the production of hepatic necrosis, a minimum number of nutrients must be present in the diet to promote the accumulation of fat in the liver. The nature of the metabolism stimulated by these nutrients provides the setting in which, at one level, proteins may be alipotropic and at another acquire lipotropic properties. Disregarding the other consequences for the organism, the fat-producing metabolism can be prevented not only by increasing the level of protein (Diet 15) but also by excluding nutrients, for example, salts (Diet 9) or salts, proteins, and fat (Diet 3). Clearly then, the salt mixture as well as yeast (Diet 10) or casein (Diet 11) are essential components of the dietary constellation promoting hepatic steatosis (compare Diets 10 and 11 with Diets 3 and 9). The role of a nutrient in the prevention of the fatty change is, therefore, a function of the metabolism promoted by the other constituents of the diet consumed by the organism.

Unless this is appreciated, unexplained paradoxical reactions are to be expected in nutritional investigations. Thus, the addition of 10 per cent food yeast to potato starch results in 95 per cent incidence of hepatic necrosis in young rats, whereas 10 per cent food yeast and 90 per cent maize meal is invariably associated with severe deformity of the bones. Obviously, the food yeast-maize combination provokes the pathological changes in the rats by virtue of the *presence* of a peculiar combination of nutrients. In other words, maize *contains* various factors which, in combination with those in food yeast, exclude necrosis as compared with those factors present in dextrinized potato starch.

By devoting exclusive attention to the deficiencies of maize, the peculiar properties of the constituents of maize are quite naturally overlooked. It is common knowledge that zein, the main protein of maize, contains hardly any lysine and practically no tryptophane. Proteins which contain considerable amounts of lysine and tryptophane are said, therefore, to be good supplements for maize. In support of this contention, the classical experiments of Hopkins and Mendel and Osborne are usually quoted. On the other hand, despite the published information, the unbalanced *amino acid content* of maize is less well appreciated. Six amino acids, namely glutamic acid, tyrosine, phenylalanine, proline, leucine, and isoleucine account for 78 per cent of the total amino acids in zein. In this connection, too, it might be added that 62 per cent of the amino acids of gliadin of wheat are made up of glutamic acid, proline, leucine, and isoleucine. This disproportionate concentration of some amino acids can have serious consequences for the organism and, undoubtedly, can acquire toxic properties in some dietary settings. Several investigators have disclosed that, in appropriate circumstances, feeding excessive amounts of one amino acid can excite severe toxic reactions in experimental animals. One need only refer to the acute necrosis of the pancreas following the exhibition of excess tyrosine (Hueper and Martin, 1943). However, even in physiological concentrations, an amino acid such as cystine may be required in some dietary settings to produce hemorrhagic nephritis or acute necrosis of the liver (Griffiths and Wade, 1939). Moreover, it has been shown that an excess of one amino acid, such as glycine, calls for an increase in the pteroylglutamic acid in the diet if the growth-inhibiting effect of glycine is to be prevented (Dinning *et al.*, 1949). That is to say, an abnormally high concentration of an amino acid sharpens the need for particular vitamins and other food factors in amounts which are out of proportion to the requirements of the organism subsisting on a different diet.

In searching for proteins to supplement maize, it is not only desirable to find protein containing sufficient amounts of lysine and tryptophane, but care should also be taken not to increase still further the already high level of glutamic acid known to be present in maize. Our experimental studies have led us to assert repeatedly that, although a minimum number of food factors is required by the organism to promote growth and differentiation, the precise requirements of the organism for any particular food factor are a function of the quality and quantity of the other constituents of the diet.

In the present state of knowledge, we may affirm that generalizations based on particular experiments about the requirements of organisms for particular food factors are not only unjustified but can be grossly misleading in the management of nutritional disease (Gillman and Gillman, 1951). Such generalizations are all the more to be condemned when they are founded on short term experiments or merely on the growth curves and survival of the animals.

Equally unjustified are the assertions of Brock and Autret (1952) that dried skimmed milk is synonymous with protein. As mentioned above, approximately 30 per cent of dried skimmed milk consists of protein. We, ourselves, have demonstrated that, whereas dried skimmed milk may assist in the rapid recovery of pellagrous infants, thus confirming the original observations of Waterlow (1948), the addition of an equivalent amount of casein had no such beneficial effect.

The constellation of factors governing the integrity of the liver and of other tissues is now so great that any claim made for the efficiency of a particular factor in safeguarding the liver and other tissues can have only a limited application. In approaching the problem of human malnutrition, it is as well to remember the timely observations of Adolph (1943), made in connection with water metabolism: "No sign from heaven is likely to point out one key that unlocks the elementary activities. The theory that a single governor exists for a physiological activity does not appear now to be substantiated. Moreover, there is no means of recognizing a regulator even if examined. A specific volley of nerve impulses or an isolatable extract are possible links in the chain, or elements in a complex. Repeatedly it is found that many factors vary simultaneously; each one is as central as any other, and only by convenience of thought is one exalted above another."

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ENZYME ACTIVITY IN FATTY LIVERS IN HUMAN INFANTS

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This paper is an account of work in progress, presented in the hope that it may give some clue to fruitful lines of attack in the future. Moreover, since the work is in a provisional stage, we shall perhaps be forgiven if more attention than usual is given to objectives and methods, rather than to actual results.

Our studies on the activity of enzymes in the human liver are based on the very simple idea that if, in man as in animals, structural damage to the liver can be caused by dietary means, that damage should be preceded by a metabolic change or biochemical lesion. If a proper perspective is to be kept, however, two points must be emphasized at the outset: First, it is apparent from the papers of Brock, Trowell, and Davies that, in the types of human malnutrition with which we are concerned, many organs besides the liver are affected. It may, therefore, be legitimate to regard liver injury not as a local phenomenon, but as a particular example of the effect of an adverse environment on glandular organs throughout the body. This aspect has been very little studied in the rat. Secondly, in the case of the liver, although the broad similarities are suggestive, there is not an exact correspondence between the types of injury produced experimentally in the rat and those found clinically in man. Fatty liver in man differs from its analogue in the rat both in pathology and in response to treatment. In man, the fat is peripheral;[1] in the rat, central.[2] This difference is surely not without significance. In man, a high-protein diet, and particularly milk, seems to be more effective in removing fat than either choline or methionine.[3] Again, although human diets in many parts of the world are low in sulphur amino acids, there is no good evidence that massive necrosis of purely dietary origin occurs in man. On the other hand, some types of liver damage in association with malnutrition or a low-protein intake, for which as yet there is no experimental analogue, have been described in man. These include pigment cirrhosis in South Africa,[4] infantile cirrhosis in India,[5] and serous hepatosis in Jamaica.[6] For these reasons we must be cautious in applying to man the results of animal experiments, a conclusion that is supported by some of the biochemical data reported in this paper.

Few people, however, would now deny that there is such a thing as nutritional liver injury in man. In the search for what the Gillmans[7] have called the "altered metabolic regulations" underlying the structural changes, it seemed best to concentrate on the dynamic aspects, rather than to measure the levels or concentrations of metabolites in the tissue. The study of enzyme activity promised to be a useful tool, more sensitive than the classical liver function tests and going nearer to the root of the problem. There may well be, of course, a great difference between the activity of an enzyme measured *in vitro* under standardized and artificial conditions, and the activity in its natural environment in the body. In the former case, we are measuring what it can be made to do, not what it actually does. This limitation for the time being must be accepted. When we use the word "activity" in this paper, we mean activity *in vitro* under stated conditions.

In theory, there are several ways in which enzyme activity or concentration might be affected by dietary deficiency. Since enzymes are proteins and, therefore, presumably in a state of rapid turnover, the rate of renewal of the enzyme molecule may be reduced if the supply of dietary protein is inadequate. There is, indeed, ample experimental evidence that deficiency of protein or of single amino acids can cause changes in the enzymatic pattern of the cell, some enzymes being reduced, other selectively preserved.[8-13] Although no clear picture with physiological meaning has yet emerged from this work, it is reasonable to suppose that, in man as in animals, dietary deficiency may cause qualitative changes, if not actual disruption, in the enzymatic machinery of the cell.

Another possible effect of malnutrition on enzymes is a kind of disuse atrophy. Potter,[14] discussing the purpose and justification of enzyme assays, wrote: "The determination of the amount of enzyme in a cell would have no value whatever, were it not for the fact that the amount of an enzyme is probably closely related to the extent of its use." He was considering the enzyme pattern in relation to cell growth and specialized function. It is only a short step from this idea to the possibility of a loss of enzyme from lack of substrate, the converse of the adaptive enzyme formation that is known to occur in bacteria, and of which there seem to be some examples in mammalian tissues.[15, 16] If such a process does occur, the effects of protein deficiency should be most apparent in those tissues which are most active in the formation or metabolism of protein. It is, perhaps, more than a coincidence that in kwashiorkor, where protein deficiency plays a prominent part, the clinical and pathological evidence shows that the organs most affected are those which are known on physiological grounds and from isotope work to have the highest protein turnover; namely, pancreas, liver, and gut. It is clear that if the synthetic mechanisms are damaged, either from lack of components or from disuse, the condition must become progressively more difficult to reverse. This may explain why such a long time is sometimes needed to restore the malnourished body to normal. Not only do the building blocks have to be supplied, but the synthetic machinery must itself be rebuilt.

Finally, enzyme activity may also be affected by shortage of essential cofactors.[17, 18] This point needs no elaboration. In this connection, however, the work of Jacquot[19] re-emphasizes the difficulty of making deductions about the behavior of the whole animal from the results of experiments *in vitro*. He has shown that, even in fatal pyridoxin or biotin deficiency, there is no change in over-all nitrogen metabolism or in the output of urea in the urine.

These are some of the speculations underlying this work. It is clear that, even if changes in enzyme activity are found, their interpretation will not be easy.

Material and Methods

All this work has been done on infants between the ages of six months and two years. The plan of investigation was simple. As soon as possible after the baby's admission to the hospital, biopsy of the liver was done by the transpleural route. The baby was then placed on a high-protein diet, consisting mainly of milk, and biopsy was repeated after some four weeks. During this time, most cases showed a marked improvement in general condition and a gain in weight, although a few were refractory and improved much more slowly.

During the past three years we have investigated cases of several different types. In some babies the clinical picture is dominated by severe weight loss, with no specific signs, and with a liver which, histologically, shows little deviation from normal. In others there is a fatty liver. This is usually accompanied by edema, with low concentrations in the plasma of total protein, albumin, and choline esterase. These cases may well be regarded as examples of kwashiorkor, although the characteristic lesions of skin and mucosae seem to be less common in Jamaica than in some parts of Africa. Yet other cases conform to the clinical and pathological picture of serous hepatosis, as described by Hill.[6] In these, there is well-marked hepatocellular degeneration in the liver. In general, however, we have avoided cases with evidence of advanced liver damage, as the interpretation of the findings is likely to be extremely difficult, and our main interest is in the detection of early changes.

In the present paper we have confined ourselves to cases with fatty liver. Even in such a restricted group, however, no two patients are exactly alike. Until much more material has been accumulated, it is not possible to treat them as a group, to average the results, and to evaluate them statistically, as in the laboratory. We have thought it best, therefore, simply to describe a few representative cases, which illustrate the results obtained so far, in spite of the danger of bias inherent in such a method. A further difficulty is that of obtaining control material from completely normal children. The best that we have been able to do is to use each case as its own control, comparing the results before and after treatment.

Details of the methods of handling the tissue and of the techniques used for the enzyme assays are given in the appendix. All the gasometric measurements have been done by the Cartesian diver technique of Linderstrøm-Lang and Holter.[20] This, in conjunction with spectrophotometric methods, makes it possible to do duplicate assays of six or seven different enzyme systems, together with estimations of fat, protein, and nucleic acids, on some 3 mg. (fresh weight) of liver. This amount of tissue is large compared with that used by some workers.[21] For the present problem, however, there is no advantage in further scaling down the amount of liver used. On the other hand, although more than 3 mg. is often available, it is unwise to count on more than this. In infants it is not possible to insert the biopsy needle as deeply as in adults, so that the pieces of tissue obtained are small. We also regard it as essential that at least half, and if possible more, should be put aside for histological examination.

Before going on to the results, it is necessary to consider two technical points of some importance. The first is the question of sampling error. Three mg. of tissue represents only about 1/150,000 of the weight of the whole liver, so that clearly our sample may not be representative. We have not felt it justifiable to try and get more than one specimen at any given time. The best, therefore, that can be done to solve this difficulty is to take two separate fragments from the same specimen (when enough tissue is available) and make two homogenates. Admittedly such fragments come from points in the liver only a few millimeters apart, but at least they represent different lobules. The histological findings suggest that the pathological processes with which we are dealing are essentially diffuse. Although there is some variation, it is from one lobule to another rather than from one large area of the liver to another. In general, such comparisons have shown good agreement between duplicate homogenates.[22]

The second difficulty is the basis of representation. Enzyme activity is usually expressed per milligram dry weight of tissue. In our material, dry weight must clearly be corrected for fat, and probably for glycogen also; but even when this is done, a true picture may not be obtained. As Kosterlitz[23] and others have shown, in malnutrition, the liver cell loses a large proportion of its cytoplasm, although the size of the nucleus remains unchanged. A diminution in enzyme activity per cell (which, presumably, is what matters) might, therefore, be masked by an increase in number of cells per unit weight. At first sight, the solution seems to be to use desoxynucleic acid as a standard of reference. In our material, however, this is open to two objections. In the first place, it is well known that there is a high degree of polyploidy in the liver. Although this is normally less marked in infants than in adults,[24] in our cases, large nuclei and binucleate or multinucleate cells are very common, appearing apparently as a response to injury. This fact destroys the basic postulate, that the DNA content per cell is constant. Secondly, it is generally assumed implicitly by biochemists that the liver is a homogeneous tissue. This may be relatively true for the rat, but it is very far from true in man. Even in apparently normal livers, as many as one third of the cells are nonepithelial cells, and this proportion may be increased in the absence of any gross pathological lesion. It is common in our cases to find infiltrations of lymphocytes in livers that are otherwise histologically normal.

For these reasons, although we have measured the DNA content of the liver in a number of cases, we cannot regard it as a satisfactory standard of reference. The best basis is probably protein nitrogen, provided there is no significant dilution with inert protein, such as collagen. Nitrogen figures are not yet available for the cases reported in this paper, and therefore the figures are expressed on the basis of fat-free dry weight, without correction for glycogen.

Results

Of the vast number of possible enzymes, only a few have so far been investigated. These have been chosen on two criteria: their physiological importance, and their concentration or activity. We cannot expect to detect changes in activity in enzymes with a QO_2 of less than 1, because this is approaching the lower limit of sensitivity of our methods. It is for this reason that we have not studied xanthine oxidase, an enzyme which seems to be particularly sensi-

Table 1

Comparison of the Activity of Some Liver Enzymes in Man and Rat

	Man[*]	Rat
Cytochrome oxidase, QO_2	12	140
Succinoxidase, QO_2	4	50
DPN-cytochrome-c reductase, QO_2	23	70
Dehydrogenases:		
lactic, QO_2	55	160
malic, QO_2	207	860
glutamic, QO_2	9	–
Transaminase, QCO_2	278	50
Choline esterase, QCO_2	8	9[†]

[*] The figures in this column are the average of results obtained in recovered cases.
[†] Female rats.

tive to adverse dietary conditions,[11, 12] because even in the rat its activity is low and in man, so far, we have not been able, in preliminary trials, to measure it with any accuracy.

Since there is almost no information in the literature, the first need is to get some idea of the pattern of enzyme activity in the normal human liver. As has been said, no strictly normal control series is available and, therefore, we use as a temporary standard of comparison, or yardstick, the average of all the figures obtained in recovered human cases. The results for eight enzymes, compared with those found by the same methods in the rat, are shown in TABLE 1. The difference in activity of the oxidative enzymes, on the one hand, and of transaminase on the other, is too great to be explained by chance variations of sampling or by errors of technique. There seems to be a real difference in enzyme pattern between the two species, a difference which raises problems of some interest. It is clearly unsound to consider each enzyme in isolation. The presence of one in high concentration must affect the activity of others. The low level of succinoxidase must, it would seem, be a limiting factor in the activity of the Krebs cycle. Transaminases can interfere with this cycle by removing or adding substrates at three stages: pyruvate, oxalacetate, and α-keto-glutarate. These substances are, as Braunstein puts it,[25] at the metabolic crossroads, and may be important links through which the activity of one enzyme system can control or affect the rate of another. At present, we do not know very much about these interactions, but the great difference that has been found between rat and man is at least a stimulus to further investigation. It suggests that the normal metabolic paths may, perhaps, not be the same in the two species, and supports the point of view already put forward, that we must not expect the effects of a given type of malnutrition to be identical.

These considerations form the background to our problem. We must now turn to the effect on activity or concentration of liver enzymes of malnutrition in general, and of that type, in particular, which causes fatty infiltration. To do this, we can draw on the experience of some 30 cases investigated in the past, but shall present, in detail, only the results obtained in three babies with severely fatty livers that have been studied recently (TABLES 2, 3, and 4).

TABLE 2

CASE 1. F. AGE 6/12. WEIGHT 5.6 KG.

	1st biopsy 9.i.53	2nd biopsy 3.ii.53	Average*
Liver fat:			
per cent of fresh weight	30.6	6.2	3
fat: nonfat solids × 100	255	30	15
Plasma protein, gm. per 100 ml.	3.7	5.7	6.5
Plasma choline esterase, Michel units	0.35	0.56	0.6
Succinoxidase, QO_2	9.7	4.2	4
Cytochrome-c reductase, QO_2	18.5	25.9	23
Dehydrogenases:			
lactic, QO_2	59	58	55
malic, QO_2	320	193	207
glutamic, QO_2	10.0	9.4	9
Transaminase, QCO_2	264	265	278

* The figures in this column are the average of results obtained in recovered cases.

TABLE 3

CASE 2. F. AGE 11/12. WEIGHT 6.6 KG.

	1st Biopsy 14.i.53	2nd Biopsy 5.ii.53	Average
Liver fat:			
per cent of fresh weight	44.7	14.2	3
fat: nonfat solids × 100	322	86	15
Plasma protein, gm. per 100 ml.	4.1	6.0	6.5
Plasma choline esterase, Michel units	0.50	0.70	0.6
Succinoxidase, QO_2	5.1	5.5	4
Cytochrome-c reductase, QO_2	17.1	30.2	23
Dehydrogenases:			
lactic, QO_2	24.3	75.0	55
malic, QO_2	176	119	207
glutamic, QO_2	6.1	18.1	9
Transaminase, QCO_2	210	307	278

TABLE 4

CASE 3. M. AGE 11/12. WEIGHT 3.5 KG.

	1st biopsy 9.x.52	2nd biopsy 20.i.53	Average
Liver fat:			
per cent of fresh weight	39.5	3	3
fat: nonfat solids × 100	335	19	15
Plasma protein, gm per 100 ml	4.5	6.2	6.5
Plasma choline esterase, Michel units	0.15	0.61	0.6
Succinoxidase, QO_2	0.6	5.2	4
Cytochrome-c reductase, QO_2	—	25.2	23
Dehydrogenases:			
lactic, QO_2	11.3	21.2	55
malic, QO_2	185	133	207
glutamic, QO_2	10.5	11.7	9
Transaminase, QCO_2	425	326	278

From these tables, it can be seen at a glance that, in so far as we are looking for a consistent change in enzyme activity in the fatty liver, the results are negative. Nevertheless, it is worth while to consider each case very briefly in turn, because although all of them had a fatty liver, they showed marked clinical differences in other respects. We shall then try to see what indications the data provide for further lines of attack.

The first case (TABLE 2) was a girl aged 6 months, moderately underweight for her age. She had edema of the feet, hands, and back, with hypoproteinemia. The liver was enlarged and fatty, but there were no other signs of malnutrition: *i.e.*, skin, hair, and mucosae were absolutely normal. Superficially, indeed, this child appeared well nourished, because she had an ample cover of subcutaneous fat, particularly on the face and limbs. She was, in fact, a perfect example of what has been called the "sugar baby." On treatment with skimmed milk powder, edema rapidly disappeared and the plasma protein concentration rose. The liver diminished in size and lost its fat. Weight gain, however, was slow. We formed the impression that body fat was being lost while muscle was being laid down. Whether or not this was the explanation, there was a great improvement in general condition in spite of the slowness of weight gain.

The second case (TABLE 3) was more severely underweight, but still retained a fair amount of subcutaneous fat. She had generalized edema, and the liver was enlarged and fatty. The striking difference between this child and the first was the presence of very severe lesions of skin and mucosae—glossitis, angular stomatitis, and desquamative dermatosis of the legs, arms, and perineum. She was a typical case of kwashiorkor, as it is seen in Africa, except that there was no dyspigmentation of the hair, no vomiting, and little diarrhea. There was a rapid response to treatment with skimmed milk; edema disappeared; and, thereafter, there was a steady gain in weight. The liver was reduced in size, and the skin lesions healed in a few days with no treatment other than milk.

Case 3 differed from the other two in being very severely underweight. Clinically, there was almost complete loss of both subcutaneous fat and of muscle, and the child seemed to consist of little more than skin and bone. The skin showed a dry scaly dermatosis, but without the desquamation leaving an underlying raw red surface, which was so prominent in Case 2. The hair, in parts, was quite white, like that of an old man. In spite of the low plasma protein concentration, there was no edema. The liver was only slightly enlarged, but it was extremely fatty, a finding which, in our experience, is unusual in total starvation or marasmus. The response to treatment was very slow, and it was only after three months in hospital that a steady weight gain began. Even before this, however, there was a gradual but undoubted improvement in that indefinable entity, the general condition. Nitrogen-balance measurements during this period showed a positive nitrogen retention, suggesting that body tissue was being built up gradually, presumably with a concurrent loss of water.

All these three cases had a great increase in liver fat. The usual method of expressing fat as a percentage of the wet or dry weight is not satisfactory. If fat, F, is calculated as a percentage of (F + S) or of (F + S + W), where S = nonfat solids and W = water, the result is misleading when F is high. It seems more illuminating to give the ratio F:S, and this we have done in the second line of the tables, taking S as 100. On this basis, the fat content in Case 3 was some 20 times normal, whereas expressed as a percentage of the wet weight it was only 13 times normal.

This degree of fatty infiltration represents a gross perversion of metabolism which is not at all reflected in the figures for enzyme activity. There was a reduction in the plasma concentration of nonspecific or pseudo-choline esterase. It has been shown in earlier work[26, 27] that, in such cases, the liver esterase is also reduced, roughly *pari passu* with that in the plasma. Low levels of liver and plasma esterase are very constantly found in malnourished infants, but this does not seem to be related in any way to the presence of absence of a fatty liver. Rather it seems to be associated with severe weight loss and general malnutrition. It is also found, as is well known, when there is advanced structural damage to the liver, as in cirrhosis.

The dehydrogenases show rather variable results. In the first biopsy, the activity of the lactic enzyme was low in two of the three cases shown. This, however, is not a constant finding. In three other cases with fatty liver, the initial levels were not low. Malic dehydrogenase, on the other hand, was present in higher concentration in the first than in the second specimen. This has been a rather consistent observation, not only in these, but in other cases also. The same is true for transaminase. In 11 out of 14 cases, transaminase activity was higher in the malnourished than in the treated liver. It would seem that both these enzymes are not only very active, but are well preserved, even selectively preserved. It would clearly be unwise, in work of this kind, to confine our interest to enzymes which are reduced by dietary deficiency. The fact that some particular enzymes are jealously retained may equally be of significance. The figures for glutamic dehydrogenase activity show much scatter, and we have not yet been able to draw any conclusions from them.

So far, not very many measurements have been made of cytochrome reductase activity. It is too soon to attach any significance to the slightly low figures found in the initial biopsies of Cases 1 and 2. The results are included here in order to demonstrate one point only: that, in Case 2, there was a fair concentration of this flavoprotein enzyme in the liver, in spite of the presence of severe signs which clinically are attributed to ariboflavinosis.

In the case of succinoxidase, the results again are variable. A very low activity, however, like that found in Case 3, has been observed in some other babies who were severely malnourished, with or without fatty liver. We cannot feel the same confidence about these results as in the case of the other enzymes, because, on two occasions, there has not been satisfactory agreement between duplicate homogenates. To admit the possibility of technical error or artifact need not, however, be entirely a confession of failure. Artifacts can be significant. The instability of the Krebs cycle enzymes probably arises from the fact that they are parts of an organized structure, parts which cannot work if their interrelationship is disturbed. Succinoxidase, although less labile than the others, is a member of this group both functionally and structurally, since it is found only in the mitochondria. In the assay of such enzymes, since some degree of organization must be preserved, we are one step nearer to conditions *in vivo* than when measuring the activity of a soluble enzyme such as transaminase. It is conceivable that the effect of malnutrition (that is, of an adverse chemical environment *in vivo*) may be not to reduce the amounts of enzyme molecules but, in some way, to destroy their organization or to make it more easily destroyed under the conditions of the *in vitro* measurements.

In the rat, most of our recent knowledge about the organization of the liver cell has been derived from the separation of particulate fractions by differential centrifugation. As far as we know, no comparable data are available for man. It seems likely, however, that, as in the case of the enzymes, the pattern of the particulates is not the same in the two species. Histologically, both mitochondria and basophil substance are much more sparse in the human liver than in that of the rat. This is confirmed by preliminary measurements that we have made of the ribonucleic acid content of the liver.[22] In our cases, this was very low. The ratio of RNA to DNA was about 1:1, compared with about 4:1 in the rat. Because of the doubt raised by Rossiter and his colleagues[28] about the specificity of the color methods, and because of the possibilities of artifact arising from working in a tropical climate without a cold room, we have postponed pursuing this investigation further until a critical examination of the methods has been completed. For this reason, we do not present here data on the nucleic acid content of the fatty liver. But the point has been raised in order to emphasize again the need for more basic information about the human liver.

In conclusion, in these fatty livers the activity of those enzymes which have been measured is, on the whole, well maintained. The only enzymes which have been found, in these and other malnourished cases, to be reduced in amount are the nonspecific choline esterase and perhaps succinoxidase. These changes seem, however, not to be related to the fatty liver *per se*, but to the general state of malnutrition of the infant. There may well be a connection between such a reduction in enzyme activity and the semi-irreversible state which some of these infants seem to reach when malnutrition has been sufficiently severe or prolonged. The fatty liver is only one element in this picture, and not necessarily the most important. Although the term "fatty liver disease"[3] has its uses (it might be a more appropriate label for Case 1 than kwashiorkor), it must not be used to imply, as one of us did five years ago,[3] that the fatty liver is the dominant feature on which a prognosis can be based. This position can no longer be held. When these babies die, in all likelihood it is not the fatty liver that kills them, but something else which, for want of a better term, we can only call generalized protein malnutrition. The severity of this does not necessarily run hand in hand with the degree of fatty infiltration in the liver.

Since there are many variables in this clinical picture, the collection of facts that can be classified, compared, and analyzed, is a slow process. We must apologize for presenting, in the meantime, a piecemeal account of work that has been done on such a small number of cases. Our justification is the hope that others will pursue this path, so that knowledge may be gained more quickly.

APPENDIX

Methods

The liver specimens are obtained with a Vim-Silverman needle inserted by the transpleural route. The infant is given paraldehyde *per rectum* beforehand, and 2 per cent novocaine is used to infiltrate the skin at the point of incision. The tissue is transferred from the needle into ice-cold saline. A piece is cut off and put into fixative for histological examination; the rest is wrapped in filter paper moistened with saline, and put into a stoppered tube which is kept on ice. This specimen is then divided into two pieces, or more if there is enough tissue to make duplicate homogenates, and each piece is weighed on a quartz fibre torsion balance. The balance that we use was constructed in the laboratory according to the principle illustrated by Strong.[29] The only modification is that we do not fuse the fibre to the beam, which is extremely difficult, but seal it with a special resin (Araldite, made by Aero Research Ltd., Duxford, Cambridge, England). This has proved very satisfactory. The zero of the balance has remained unchanged for 18 months. The only disadvantage of this method is that it is not possible to coat the balance with platinum by painting with chloroplatinic acid and flaming in order to prevent the accumulation of electrostatic charges. However, at the level of sensitivity at which we work, and in the rather humid climate of the tropics, we have not had any trouble from this source. We have not aimed at a sensitivity greater than 2 μg. With 2 mg. of tissue the error of weighing is still much less than the other errors of the chemical or enzymatic measurements.

One piece, after weighing, is put over phosphorus pentoxide to dry, and reweighed the next day, to give the water content. This piece should if possible weigh at least 1 mg., fresh weight, or ¼ mg. dry weight, although the chemical measurements can be done with less. The dried liver is put into a micro-Soxhlet apparatus similar to that described by Kirk,[30] extracted with hot ether, dried, and weighed again. This method of measuring fat content by difference, after ether extraction, gives results that are a little low. It is certain that some of the phospholipid is left behind. In the normal liver, this method gives a fat content of about 3 per cent of the wet weight, compared with the accepted normal value of 5 per cent. The difference is consistent, however, and is of little importance for our purpose, since the neutral fat does seem to be taken out quantitatively.

In the past, we have used this dried defatted tissue for measurements of nucleic acids and of protein nitrogen. The dry specimen was homogenized, and extracted with hot trichloracetic acid, according to the method of Schneider.[3] The final volume of extract was about 30 cu. mm. Total nucleic acids were measured in the extract by ultra-violet absorption after diluting 25 times with water. In this way, the TCA blank becomes negligible. DNA was measured by the diphenylamine reaction. As this reaction is not very sensitive, it was necessary to have a final volume of not more than 20 cu. mm., and this involved using a microscope colorimeter.[22] RNA was measured by difference. After extraction with TCA, protein nitrogen was measured in the residue by digestion and nesslerization.

The other piece of tissue is used for the enzyme assays, and should weigh not less than 2 mg. We homogenize in a volume of 20 cu. mm., and aim at a tissue concentration of about 10 per cent. If less than this is used, the accuracy of measurement suffers for those enzyme systems, like succinoxidase, which have a low QO_2. The tissue is homogenized in ice-cold 0.88M sucrose in a tube 2 to 3 mm. in diameter. The pestle of the homogenizer is made of glass and is rotated by a motor. The conditions of homogenization are kept as standardized as possible. During homogenization, which lasts only for 30 seconds, the tube is held in a large brass block previously cooled on ice.

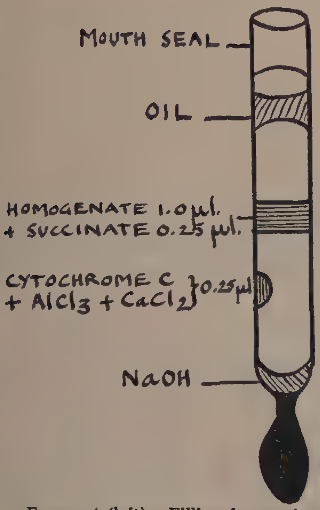
Once the homogenate has been made, it is necessary to work quickly. Of the enzymes investigated so far, we have not found any loss of activity in *intact* tissue that has been left on ice for several hours; but in a homogenate, some enzymes do become inactivated at an appreciable rate on standing, notably succinoxidase. The dehydrogenases, transaminase, and choline esterase seem to be stable for many hours. Immediately after the homogenate has been made aliquots are removed and diluted for the assays of dehydrogenases and of cytochrome-c reductase. For the former, the dilution is made in water, with a tissue concentration of about 5 μg. per cu. mm. For the latter, the dilution is made in 0.88M sucrose, with a tissue concentration of about 3 μg. per cu. mm. Two sets of measurements can then be done simultaneously, one by spectrophotometric, the other by gasometric methods. All the gasometric measurements have been made by the Cartesian diver technique of Linderstrøm-Lang and Holter, as modified by Waterlow and Borrow,[32] and by Borrow and Penney.[33] This technique is sensitive, accurate, and versatile, and the apparatus needed is very simple to make. Its main limitation is the time needed to fill the divers. On the average we have not found it possible to fill and measure more than about two divers an hour. If each is run for 30 to 60 minutes, the limitations of fatigue make it difficult for one person to run more than about 16 divers in one day.

Assay Methods

Gasometric. All assays have been done at least in duplicate. In the first instance, the conditions chosen have been those which have been worked out for assays on rat liver. Whenever enough time and enough tissue have been available, measurements have also been made with varying concentrations of substrate, buffer, *etc.*, since it cannot be assumed that the optimum conditions are the same for man and the rat.

Succinoxidase. The method was that of Schneider and Potter.[34]

Diver filling (FIGURE 1). *Bottom drop:* 0.5 μl. of 2N NaOH. *On the side-wall:* 0.25 μl. of a mixture containing cytochrome c (Wyeth) 2.5 mg. per ml.; $AlCl_3$ 3×10^{-3}M; $CaCl_2$ 2.5×10^{-3}M. *Homogenate:* 1 μl. of sucrose homogenate, strength about 10 per cent. To this was added 0.25 μl. of a mixture con-



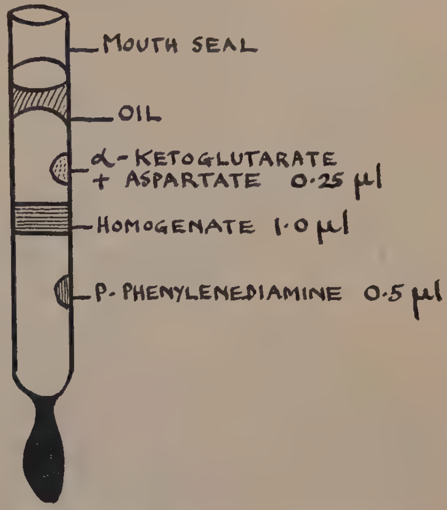


FIGURE 1 (left). Filling for succinoxidase assay. FIGURE 2 (right). Filling for transaminase assay.

taining Na succinate 0.5M, phosphate buffer, pH 7.4 0.125M. This addition was made during the filling of the diver.

After equilibration, the homogenate plus substrate was mixed with the side-drop containing cytochrome.[32] Readings were made for one hour. A blank was always run containing all reagents except succinate, because at this high tissue concentration the endogenous respiration is appreciable ($QO_2 = 1$ to 2).

Transaminase. Our method is essentially that of Ames and Elvehjem,[35] in which the reaction measured is the formation of glutamic and oxaloacetic acids from aspartic and α-keto-glutaric acids. The oxaloacetate is then decomposed, and the CO_2 output measured. It was found, however, that activity was often not linear in relation to the amount of tissue, being less with large amounts. The same effect was found when the time of incubation was varied, the activity being relatively less with incubations of 15 or 20 minutes than of 5 or 10 minutes. It is probable that this arises from the position of the equilibrium point in this reaction, which is reached when about ⅓ of the aspartate and α-keto-glutarate have reacted.[36] The effect has been reduced, but not eliminated, by increasing the concentration of substrates. The strength of the homogenate has been kept as constant as possible and measurements have been made in duplicate with only one strength of tissue incubated for 5 and 10 minutes. For decomposing the oxaloacetate we have used a solution of p-phenylenediamine instead of aniline citrate. With this, evolution of CO_2 is complete in about 5 minutes. Aniline citrate has the disadvantage of being oily, so that it cannot be placed as a discrete drop on the diver wall.

Diver filling (FIGURE 2). *Lower drop:* 0.5 µl. of M/1 p-phenylenediamine in N/1 HCl. *Homogenate:* 1 µl. of homogenate diluted to 2 per cent with 0.88M sucrose. *Upper drop:* 0.25 µl. of a mixture containing aspartate 0.3M, α-keto-glutarate 0.3M, phosphate buffer 0.15 M, pH 7.4. *Homogenate and* *upper drop* mixed by applying a negative pressure after equilibration. Lower drop mixed by applying a positive pressure 5 or 10 minutes later. CO_2 output is complete in 5 to 10 minutes.

For the blank exactly the same filling is used, but the two mixings are carried out in immediate succession so that the incubation time is zero.

Choline esterase. Diver filling. 1.25 μl. of homogenate. To this is added during the filling of the diver 0.25 μl. of 0.19M $NaHCO_3$ and 0.3 μl. of 2.5 per cent acetyl choline. Gas-phase: 5.2 per cent CO_2 in oxygen. Divers gassed under water by the technique of Waterlow and Borrow.[32] Readings are taken from 15 to 45 minutes after the moment of adding the acetyl choline.

Spectrophotometric Methods

Dehydrogenases. The lactic, malic, and glutamic dehydrogenases were measured by following the reduction of DPN. Density measurements were made at 340 mμ with the Beckmann spectrophotometer. The details of the method were kindly supplied by Dr. J. S. Strominger of the National Institutes of Health. The amounts of tissue used for the assays were of the order of 5 μg. for the lactic dehydrogenase, 2 μg. for the malic dehydrogenase, and 50 μg. for the glutamic dehydrogenase. The final volume was, in all cases, 0.110 ml. Results are expressed in terms of QO_2. This calculated from the observed density changes by the use of Ohlmeyer's figure of 6270 for the molecular extinction coefficient of $DPNH_2$.[37]

DPN-Cytochrome-c Reductase. This enzyme was measured by a micro-adaptation of the method of Brody *et al.*[38] In this method the amount of reduced cytochrome c is determined by measuring the optical density at 550 mμ. The amount of tissue used was of the order of 50 μg. and the final volume was 0.110 ml. Results are expressed as QO_2 values, which were calculated from the observed density changes by the use of the figures 9,560 and 28,100 for the molecular extinction coefficients of oxidized and reduced cytochrome c.[39]

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THE QUESTION OF THE RELATIVE IMPORTANCE OF PROTEIN AND LABILE METHYL IN THE DEVELOPMENT OF FATTY LIVER AND CIRRHOSIS IN MAN

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The hypothesis that fatty liver disease and cirrhosis in man is caused by nutritional deficiency has never been proved,[5, 10] but many clinicians working in temperate climates hold the view that the lesion develops in alcoholics when they cease to ingest a good mixed diet. Investigators working in the tropics believe that a low intake of protein is an important factor in the causation of "kwashiorkor" in children.[19, 23, 2] Trowell[20] has stated that animal protein is particularly important in this respect.

Animal experiments have shown that at least part of the lipotropic effect of protein is the resultant of the opposing influences of cystine and methionine, that a deficiency of choline leads to fatty liver, and that hepatic fibrosis results from chronic interference with the utilization of methionine.[9] Other nutrient interrelationships are involved in the production of fatty liver in animals. Cystine exerts a positive effect on the accumulation of liver fat only if the diet is low in niacin,[22] and vitamin B12 and folic acid have been shown to influence the metabolism of choline and methionine.[15] Some evidence suggests that unidentified factors may be present in the protein molecule which affect liver fat deposition.[21, 3, 1] A factor, as yet unidentified, has recently been purified from casein and from certain yeasts which protects rats against dietary necrotic liver degeneration.[17, 18]

The present communication deals with the incidence of hepatomegaly, fatty liver, and cirrhosis in three West African tribes and in one group of racially similar wealthy native traders, as related to their diets and other environmental factors.

Methods and results. A general description of the investigations carried out in Nigeria between 1947 and 1951, and the methods used to determine nutrient intakes of Nigerian natives, have been published elsewhere.[12, 13] The samples of the tribes were formed of all the inhabitants estimated to be over twelve years of age in three small villages in the tribal areas. The traders lived in a town of some size in the center of the Niger Delta. Clinical enlargement of the liver was diagnosed if the organ was palpable two fingers below the costal margin in the midclavicular line, the subject lying on his back and breathing normally. Ascites and jaundice were encountered occasionally in association with hepatomegaly, gynecomastia and feminism were seen occasionally in males, spider naevi were noted in a few natives who had pale skins, and muscular wasting was a prominent feature of the more advanced cases. Hepatomegaly was found at all ages, but more frequently after the third decade. The lesion was observed more often in females than in males, but the difference was not statistically significant. Infective hepatitis is a common disease throughout Nigeria. Such cases of liver enlargement were excluded from this study.

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* I am grateful to the Inspector General of Medical Services, Nigeria, for permission to publish this paper, and to Doctor Klaus Schwarz, National Institutes of Health, U. S. Public Health Service, Bethesda, Md., for help in its preparation.

Specimens of liver tissue were obtained by needle biopsy or by laparotomy from many individuals suffering from hepatomegaly, the commonest histological picture being that of portal cirrhosis, varying amounts of fat being present in the parenchyma; but bizarre appearances and destruction and regeneration of liver tissue were observed, and on two occasions schistosome ova were seen in the sections.

The incidence of hepatomegaly in the four groups, together with the prevalent parasitic infestation rates, the calculated daily intake of alcohol, and the frequency of a past history of transient jaundice are shown in TABLE 1. The consumption of alcohol, a habit freely indulged by men, women, and often children, is highest in the traders, in which group the incidence of enlarged liver is least. The total parasitic load is approximately equal in the three tribes, and it is believed that nutritional demands made, and any hepatic damage caused, by parasitic toxins or the parasites themselves, are likely to be of the same degree in each tribe. Clinical disease and pathological states attributable to specific parasites are seen less often than would be expected from the numbers of natives who carry these organisms. The incidence of hepatomegaly is

TABLE 1

THE INCIDENCE OF HEPATOMEGALY, THE PREVALENT PARASITIC INFESTATION RATES, THE DAILY ALCOHOL CONSUMPTION, AND THE FREQUENCY OF A PAST HISTORY OF TRANSIENT JAUNDICE IN THREE WEST AFRICAN TRIBES AND IN ONE GROUP OF WEALTHY TRADERS

	Traders (90)[*]	*Ijaws* (112)[*]	*Isokos* (123)[*]	*Dakarkerris* (104)[*]
	%	%	%	%
Incidence of hepatomegaly	3	8	19	9
Parasites:[‡]				
Schistosomes	1	0	2	43
E. histolytica	4	8	3	3
Ankylostomes	73	96	95	65
Ascaris	17	20	31	5
G. lamblia	3	4	2	6
Dracontiasis	0	0	0	7
Filaria				
loaloa	4	4	3	0
bancrofti	0	0	0	3
Splenic index (children 2–10 years)	17	17	22	17
Previous history of transient jaundice	3	8	6	6
	ml.	*ml.*	*ml.*	*ml.*
Alcohol consumption:—average daily intake of:				
Home brewed beer (2–5%)	0	0	0	228
Palm wine (1–7%)	120	103	250	0
Local spirits (60–70%)	20	107	13	0
Imported beer (4–6%)	100	0	0	0
Imported spirits (50–70%)	64	0	0	0

[*] Numbers examined clinically.

[†] Figures in parentheses denote alcoholic content.

[‡] Number of individuals found to be carriers of parasite or its ova, not all suffering from specific disease as a result of the infestation.

TABLE 2

CALCULATED DAILY INTAKE OF NUTRIENTS OF THREE NIGERIAN TRIBES AND OF ONE GROUP OF WEALTHY TRADERS

Group	Protein		Fat	ChO	Vitamin A*	Thiamine	Riboflav.	Niacin	Vit. C	Fe	Ca	Cals.
	An.	Veg.										
	g.	g.	g.	g.	I.U.	mg.	mg.	mg.	mg.	mg.	mg.	
Traders	50	34	86	473	9200	1.02	1.37	18.1	71	21	1302	3000
Ijaws	68	12	39	379	5700	0.30	0.68	12.1	40	24	2800	2200
Isokos	20	26	43	424	7400	0.69	0.65	8.9	30	21	664	2300
Dakarkerri	2	85	24	595	700	3.70	0.98	25.0	34	57	581	2900

	Contribution to calories (%)			Composition of diets by weight (%)		
	Protein	Fat	Carbohydrate	Protein	Fat	Carbohydrate
Traders	11.2	25.8	63.0	13.1	13.4	73.5
Ijaws	14.6	16.4	69.0	16.1	7.8	76.1
Isokos	8.1	17.1	74.8	9.0	8.4	82.6
Dakarkerri	11.8	7.3	80.9	12.3	3.4	84.3

* 1 i.u. of Vitamin A taken as equivalent to 3 i.u. of Vitamin A in the form of carotene.
No allowance made for destruction of nutrients by cooking.

least in the traders (3 per cent) and greatest in the Isoko (19 per cent), the corresponding figures for the Ijaws and Dakarkerris being 8 per cent and 9 per cent respectively, the difference between the rate in the Isoko and in the other two tribal groups being significant (P = 0.01). A correlation between hepatomegaly on the one hand and a previous history of jaundice or any parasitic infestation on the other hand was not found.

Full details of the foodstuffs eaten are given in the appendix. The calculated daily intake of nutrients is given in TABLE 2. It will be seen that the amounts of individual nutrients vary widely from group to group, resulting from the use of different staple foods in the different regions of Nigeria. The traders' diet is considered to be reasonably adequate, but the three tribal diets each show grave deficiencies of one or more nutrients. A low intake of protein, however, is the only feature by which the Isoko diet is distinguished from the diets of the other two tribes. The Isokos ingest 46 gm. of protein daily, of which 20 gm. is derived from animal sources; the Ijaws' diet contains 80 gm. of protein, 68 gm. being of animal origin; the Dakarkerris eat 87 gm. of protein, of which all but 2 gm. is of vegetable origin.

The essential amino acid composition of the diets was calculated using numerous analyses of the amino acid content of foodstuffs, choosing an average according to species, soil, and climate. For these analytical data, I am indebted to Doctor M. Autret of the Food and Agricultural Organization. Sufficient data applicable to African foodstuffs has not been found from which to assess the cystine content of the diets. The results, together with the amounts of protein nitrogen ingested,[4] are given in TABLE 3. Comparison is made between the essential amino acid composition of the Nigerian diets and the requirements of man advocated by Rose.[16] TABLE 3 also includes an estimate of

TABLE 3

THE CALORIC NITROGEN, PROTEIN ESSENTIAL AMINO ACID, AND CHOLINE COMPOSITION OF THE DIETS OF THREE NIGERIAN TRIBES AND OF ONE GROUP OF WEALTHY NATIVE TRADERS. COMPARISON MADE WITH THE RECOMMENDED DAILY INTAKES OF ESSENTIAL AMINO ACID (ROSE)

Nutrient	Rose (1949)		Traders	Ijaws	Isokos	Dakarkerris
	Minimum*	Allowance				
Calories	3600	3600	3000	2200	2300	2900
Protein (g.)						
Animal			50	68	20	2
Vegetable			34	12	26	85
Nitrogen† (g.)	10	10	13	12	7	13
Essential amino acids (g.)	6.4	12.8	30.3	28.8	15.8	27.2
Iso-leucine	0.7	1.4	4.1	3.8	2.0	3.6
Leucine	1.1	2.2	6.5	5.8	3.2	8.3
Lysine	0.8	1.6	5.3	5.8	2.7	2.1
Methionine	1.1	2.2	2.0	2.2	1.2	1.3
Phenylalanine	1.1	2.2	3.5	3.0	1.9	3.4
Threonine	0.5	1.0	3.4	3.3	1.8	3.1
Tryptophan	0.3	0.5	1.0	0.8	0.6	0.9
Valine	0.8	1.6	4.5	4.1	2.4	4.5
Choline (g.)			0.92	0.65	0.60	0.65
Incidence of hepatomegaly (%)			3	8	19	9

* Minimal requirements for maintenance of nitrogen in healthy young American adult males.
† Does not include any nonprotein nitrogen present in the foodstuffs used.

the choline content of the African diets, which has been made from such published data as are available.[6, 7, 8, 11] For some of the foods, the choline and amino acid contents had to be derived by analogy.

If Rose's daily recommendations are considered, the only deficiencies of essential amino acids detectable are those of methionine in the diets of the traders, Isokos and Dakarkerris, and of phenyalanine in the Isoko diet. Of the eight essential amino acids, each group ingests more than Rose's minimal requirements for the maintenance of nitrogen balance in healthy adult American males. The Isokos attach great importance to the capture of the small animals which provide them with 20 gm. of animal protein,[14] and it is of interest that this amount is sufficient to raise the methionine in their diet to approximately the same level as that of the Dakarkerri diet; namely, 1.2 gm. per day. The vegetable proteins are reported to be low in cystine;[24] therefore, good reason does not exist to suppose that the Dakarkerri diet contains considerably more cystine than that of the Isokos.

Discussion. The data presented above show that the only feature whereby the Isokos can be distinguished from the other two tribal groups is by a low intake of protein. The Dakarkerris and Isokos ingest equally low amounts of methionine, but there is no good reason to suppose that either diet is deficient in labile methyl groups in view of the estimated choline contents of the two diets.

Enlargement of the liver undoubtedly results from invasion of the organ by parasites, as in amebic hepatitis and schistosomiasis, and part of the total incidence of hepatomegaly in all groups is due to such causes; but these lesions are manifested by focal necrosis and abscess formation, patchy fibrosis and associated regeneration of liver tissue, not by fatty infiltration and uniform cirrhosis. A large percentage of the sections of liver from individuals suffering from hepatomegaly in this series showed the latter picture. It has been pointed out above that the incidence of parasitic infestations does not parallel the incidence of hepatomegaly, and it is believed that nutritional factors account for the varying incidence of this clinical feature in the tribes under consideration.

In view of the above discussion, and as a low intake of protein is the only feature by which the Isoko can be distinguished from the other groups considered, a further argument is advanced to explain the high incidence of hepatomegaly in this tribe: namely, that some unidentified factor, which occurs in, or is associated with, protein is involved in the protection of the liver from fatty infiltration and cirrhosis, and possibly also from the effects of parasitic toxins and alcohol. The existence of such a factor has been suggested in the literature cited in the introduction to this paper. Such a factor would appear to be present and active both in vegetable and animal protein and, therefore, is unlikely to be related to vitamin B12.

Summary. (1) The incidence of hepatomegaly (as defined) in three Nigerian tribes and in one group of wealthy native traders is recorded, together with a description of the diets eaten, the histological appearance of the liver lesions, the prevalence of parasitic infestations, and the alcohol consumption of each group.

(2) The importance of dietary protein in the etiology of the disease is discussed. The evidence obtained does not support the view that lack of any of the essential amino acids or lack of labile methyl in the diet is of importance in the development of fatty liver and cirrhosis in the groups investigated.

(3) It is suggested that some unidentified factor, which occurs in association with both animal and vegetable protein, plays a part in preventing the development of fatty liver and cirrhosis in man.

APPENDIX

AVERAGE DAILY FOOD CONSUMPTION OF NIGERIAN PEASANTS AND TRADERS (ADULTS)

Foodstuff	Scientific name	Traders	Ijaws	Isokos	Daks.
		g.	g.	g.	g.
Wheat flour (70%)	Triticum vulgare Host Cultigen	70	—	—	—
Rice, milled	Oryza sativa Linn.	76	—	—	—
Rice, home pounded	Oryza sativa Linn.	—	—	—	0.3
Maize mature yellow	Zea mays Linn.	30	29	15	—
Maize immature		10	—	7	14
Guinea corn	Sorghum vulgare Linn.	—	—	—	765
Bulrush millet	Penisetum typhoideum Rich.	—	—	—	22

APPENDIX (*continued*)

AVERAGE DAILY FOOD CONSUMPTION (*continued*)

Foodstuff	Scientific name	Traders	Ijaws	Isokos	Daks.
		g.	g.	g.	g.
Cassava:	Manihot utilissima Pohl.				
flour		109	186	143	5
starch		102	92	172	—
"fufu"		—	—	63	—
fresh tuber		—	77	—	—
Yam	Dioscorea sativa Linn.	300	63	633	—
Cocoyam	Colocasia antiquorum Schott.	—	12	—	4
Kaffir potato	Plectanthus sp.	—	—	—	15
Plantain, green	Musa paradisica Linn.	17	13	14	—
Banana	Musa sapientium Linn.	22	—	—	—
Sweet potato	Ipomoea batatas Linn.	—	19	—	—
Cow pea	Vigna sinensis	15	—	13	15
Jak fruit	Artocarpus integer Merr.	—	—	3	—
Groundnuts	Arachis hypogaea Linn.	4	—	2	—
Locust bean cake	Parkia filicoidea Welw.	—	—	—	1
Pumpkin seed	Cucurbita spp.	3	—	1	—
Coconut kernel	Cocos nucifera Linn.	2	—	—	—
Oil bean kernel	Pentaclethra macrophylla Bth.	—	—	1	—
Dika nut kernel	Irvingia gabonensis Baill	1	—	—	—
Kola nut kernel	Kola accuminata Schott. & Endl.	3	1	1	1
Shea nut husk	Butyrospermum parkii	—	—	—	45
Shea nut oil		—	—	—	1
Onions	Allium cepa Linn.	4	—	—	—
Okra, fresh	Hibiscus esculentus Linn.	6	—	—	2
Okra, dried		—	—	—	1
Red sorrel, fresh	Hibiscus sabdariffa	—	—	—	1
Red sorrel, dried		—	—	—	5
Green leaves	Corchorus spp, amaranths and others	12	—	—	—
Peppers,	Capsicum frutescens Bl.				
red:					
dry		3	4	3	2
fresh		2	1	3	—
green fresh		5	1	2	—
Baobab leaf, fresh	Adansonia digitata B. Juss.	—	—	—	16
Baobab fruit		—	—	—	1
Tomato, fresh	Lycopersicum esculentus Mill.	4	—	2	—
Avocado pear	Persia americana Mill.	26	—	—	—
Mango	Mangifera indica Linn.	20	—	2	45
Taura	Detarium senegalense Gmel.	—	—	—	1
Guava	Psidium guajava Linn.	4	—	—	—
Pawpaw	Carica papaya Linn.	12	—	—	—
Orange	Citrus sinensis sp.	10	—	—	—
Custard apple	Annona reticulata Linn.	7	—	—	—
Fish, mixed estuary and river:					
dried		28	78	18	1
fresh		62	165	18	—
Beef		96	—	—	—
Mutton		12	—	—	—

APPENDIX (*continued*)

AVERAGE DAILY FOOD CONSUMPTION (*continued*)

Foodstuff	Scientific name	Traders	Ijaws	Isokos	Daks.
		g.	g.	g.	g
Pork, fat		16	—	—	—
Goat		—	—	6	—
Monkey, dried	Various species	—	—	17	—
Rat meat	Cricetomys gambianus	—	—	2	2
Rat meat	Thryonomys swinderianus	—	—	1	—
Pangolin	Manis longicaudata	—	—	3	—
Porcupine	Atherurus africanus	—	—	2	—
Frogs' legs	Rana mascarenensis	—	—	3	—
African snail	Archachatina sp.	3	—	3	—
Palm weevils	Rhyncophorus phoenicis	1	—	1	—
Snake meat	various species	—	—	—	0.3
Shrimps, dry		2	1	—	—
Prawns, fresh		—	4	—	—
Oysters, local		—	7	—	—
Liver		4	—	—	—
Eggs		10	—	—	—
Caterpillars		—	—	—	4
Sugar cane stem	Saccharum officinarum Linn.	26	4	3	6
Palm oil	from Elaeis guineensis Jacq.	36	27	35	—
Margarine, imported		5	—	—	—
Jam, imported		8	—	—	—
Sardines, tinned		3	—	—	—
Milk:					
sweetened condensed		5	—	—	—
evaporated		28	—	—	—
local, sour		—	—	—	16
Palm wine (ml.)	from Elaeis guineensis and Raphia vinifera	120	103	250	—
Native beer (ml.)	from sorghum vulgare and penisetum	—	—	—	228
"Illicit gin" (ml.)		20	107	13	—
Gin, imported (ml.)		26	—	—	—
Whiskey (ml.)		23	—	—	—
Brandy (ml.)		15	—	—	—
Beer, imported (ml.)		100	—	—	—
Tea		4	—	—	—
Coffee		3	—	—	—
Nut (Urhobo-"ewoie")		—	1	1	—
Native salt		4	4	3	1
Imported salt		10	7	5	3

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RELATIVE EFFECTS OF PROTEIN AND LIPOTROPIC SUBSTANCES IN THE TREATMENT OF NUTRITIONAL CIRRHOSIS IN RATS*

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It is established that rats fed diets low in protein and deficient in lipotropic substances develop fatty changes and cirrhosis of the liver. It has also been shown that the addition of choline and methionine to these diets prevents the development of the lesions. Fewer studies have dealt with the treatment of nutritional cirrhosis once it is fully developed.

The first reports on treatment of dietary cirrhosis in the rat were those of Lowry, Daft, Sebrell, Ashburn, and Lillie.[1, 2] After producing cirrhosis in weanling rats with a diet containing 4 per cent casein, liver biopsies were performed, after which the animals were placed in two therapeutic groups, one receiving the basal diet plus 40 mgm. of choline daily, the other receiving a high casein diet containing either 30 or 50 per cent casein. In a short term study, animals were sacrificed at intervals up to 42 days and, in a longer term study, at intervals up to one year. Comparison was made with the initial biopsies. In both groups, the addition of choline to the basal diet and the change to a higher casein diet brought about disappearance of fat, regeneration of liver cells, but no apparent decrease in the amount of connective tissue.

Sellers, Lucas, and Best[3] tested various dietary regimens on young adult rats with cirrhosis of the liver induced by carbon tetrachloride poisoning. After a moderate degree of cirrhosis was produced, a representative sample was sacrificed for control studies, the remainder being placed on the test diets for 12 to 13 weeks. In these experiments, the addition of choline, of methionine, or an increased proportion of casein brought about disappearance of fat, regeneration of liver cells, and loss of connective tissue as well. These agents seemed to be equally effective. It was concluded, therefore, that the reparative effects were attributable largely to lipotropic substances.

In a more recent report by György and Goldblatt,[4] employing a large series of rats, doubt was thrown on the effectiveness of lipotropic substances, more particularly in advanced stages of the disease. Because of the high mortality involved, biopsy technique was abandoned, and their observations were based largely upon sampling after 75 to 150 days of treatment. In those animals with moderate degrees of cirrhosis, lipotropic substances seemed to exert a curative effect, whereas in more advanced cirrhosis, the addition of these substances to the basal diet was much less effective. Best results were obtained by a combination of casein and liver extract.

The problem was re-examined because of the divergent results. In the experiments to be described, nutritional cirrhosis was produced in the rat by a diet patterned after that of Daft, Sebrell, and Lillie.[5] An attempt was made to evaluate the effects of dietary protein and of lipotropic factors separately and together in reversing the disease process. These studies suggest that high protein feeding produces reparative effects greater than those attributable to choline and methionine.

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* Permission has been granted by the Journal of Experimental Medicine to reprint TABLES 1, 2, and 3 in the present report.

Albino rats of the Sherman strain, 6 to 8 weeks in age, were employed. In a preliminary study with 55 animals, both male and female rats were employed. After cirrhosis was produced by a 4 per cent casein diet, the rats were divided in groups, one of which received the basal diet plus choline, whereas the other received a 30 per cent casein diet without supplementary choline. It became apparent that the male was more susceptible to cirrhosis than the female (as pointed out by others) and that sampling was not a dependable technique in a series of this size. It was, therefore, decided to employ only male animals and biopsy technique, despite the attendant mortality.

The composition of the experimental diets is shown in TABLE 1. The Hubbell-Mendel salt mix was employed. The rats were fed daily supplements of thiamine chloride, riboflavin, pyridoxine, calcium pantothenate, and niacin, and weekly supplements of percomorph oil and alpha-tocopherol. The animals were permitted to feed *ad libitum.*

The plan of the experiment is shown in TABLE 2. Choline was mixed with the diet in a proportion of 0.5 per cent. The average daily consumption per rat was 40 mgm. of choline. Methionine was added at a level of 0.8 per cent. The average daily consumption per rat was 64 mgm. This was estimated to be equivalent to the methionine content of the 30 per cent casein diet.

Results. The caloric intake was essentially the same for those on the 4 per cent and 30 per cent casein diets, but their rates of growth varied markedly,

TABLE 1

COMPOSITION OF EXPERIMENTAL DIETS

Low-protein		High-protein	
	per cent		*per cent*
Vitamin-free casein	4.0	Vitamin-free casein	30.0
L-cystine	0.5	L-cystine	0.5
Wesson oil	5.0	Wesson oil	5.0
Salt mixture	3.0	Salt mixture	3.0
Corn-starch	87.5	Corn-starch	61.5

TABLE 2

PLAN OF EXPERIMENT

Group no.	Foreperiod (16–19 wks.)	Treatment period (15–19 wks.)	
1	4 per cent casein diet	Biopsy	4 per cent casein diet + choline + methionine
2	4 per cent casein diet	Biopsy	30 per cent casein diet + choline
3	4 per cent casein diet	Biopsy	30 per cent casein diet
4	4 per cent casein diet (control)	Biopsy	4 per cent casein diet
5	30 per cent casein diet (control) + choline	Biopsy	30 per cent casein diet + choline

those on the 30 per cent casein diets showing a sharp acceleration of growth. Unexplained was an intercurrent dermatitis, particularly among those on the 30 per cent casein diet. The lesions resembled those related to deficiency of unsaturated fatty acid. However, on adding linoleic acid to the diets, the dermatitis was but partially arrested. Of 143 rats employed, 69 died during the foreperiod. In this early stage, death was due chiefly to hemorrhagic cortical degeneration of the kidneys, presumably from choline deficiency. Nine others died within one week of the diagnostic biopsy. There remained 70 rats suitable for testing various therapeutic diets.

During the period of treatment, there was a sharp reduction in death rate in all groups, once the animals survived a critical period of about two weeks. The death rate was higher in those fed the 4 per cent casein diet supplemented with choline and methionine than in those fed 30 per cent casein diets.

Comparison was made of histologic sections obtained at the time of biopsy and at autopsy. Only those specimens were compared in which animals received treatment for at least 10 weeks. Sixty animals satisfied this criterion.

The results are shown in TABLE 3. The tabulation is restricted to fatty infiltration and changes in connective tissue, since other changes are difficult to compare in a quantitative fashion. Certain trends seem evident. For example, a sharp reduction in fatty infiltration was seen when 4 per cent casein diet was supplemented with choline and methionine. A still greater lipotropic effect was seen in livers of rats whose diets were changed from 4 per cent to 30 per cent casein, with or without added choline. Fibrosis appeared to increase in rats on the 4 per cent casein diet, despite the addition of choline and methionine. In contrast, those rats whose diets were changed from 4 to 30 per cent casein showed either moderate regression (group 2) or arrest (group 3) of this process. In this small group, it is doubtful whether the differences between

TABLE 3

HISTOLOGIC APPEARANCE OF LIVER AS DETERMINED FOR SPECIMENS AT BIOPSY AND AUTOPSY*

Group No.			Fatty infiltration		Connective tissue	
			Biopsy	Autopsy	Biopsy	Autopsy
1	4 per cent casein + choline + methionine (18 rats)	Absent	0	4	2	2
		Minimal	3	10	8	3
		Abundant	15	4	8	13
2	30 per cent casein + choline (21 rats)	Absent	2	10	1	7
		Minimal	8	11	13	7
		Abundant	11	0	7	7
3	30 per cent casein alone (12 rats)	Absent	0	7	1	1
		Minimal	3	4	6	6
		Abundant	9	1	5	5
4	4 per cent casein control (4 rats)	Absent	0	0	1	0
		Minimal	0	0	2	0
		Abundant	4	4	1	4
5	30 per cent casein control (5 rats)	Absent	5	5	5	5
		Minimal	0	0	0	0
		Abundant	0	0	0	0

* Only those specimens are compared in which animals received dietary treatment for 10 or more weeks.

group 2 and group 3 are significant, whereas the differences between group 1 and either group 2 or group 3 appear significant.

In pursuing this problem further, preliminary studies have been made by Dr. Ernest Schmattolla with a mixture of amino acids replacing the 30 per cent casein diet. The composition of the amino acid mixture was similar to the amino acid composition of casein. Such amino acid mixtures appeared to be as effective in promoting repair of the liver as any of the diets employed above.

Evaluation of results is always difficult, and comparison with other studies is hazardous because of different experimental conditions. There are several possible explanations. Possibly methionine contained in the 30 per cent casein diet is more effectively utilized than when it is added as a supplement to the low casein diet. Possibly other amino acids play a rôle in the reparative process.

In conclusion, within the conditions of these experiments, the studies suggest that the feeding of diets containing 30 per cent casein to rats with nutritional cirrhosis produces reparative effects greater than those provided by diets containing 4 per cent casein with supplementary choline and methionine.

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Discussion of the Paper

Doctor P. György, Philadelphia: It is too much to ask to expect identical results with beneficial prophylactic measures in the therapy of the same condition, such as cirrhosis. In prophylactic experiments, the effective factors should and will prevent the development of the major specific metabolic and anatomical changes. Therapeutic efforts, on the other hand, have to deal with the arrest or even regression of already existing metabolic and anatomical disturbances. For instance, in the case of cirrhosis, the progress of fibrosis has to be checked or even reversed, in addition to the repair of all other concurrent pathologic manifestations. Furthermore, in cirrhosis, not only the liver but often other organs as well, such as the kidney and endocrine organs, among the latter especially the gonads, are found to be involved in the over-all disease. In decompensated liver disease, high-protein diet may even be injurious just as exercise may be harmful in decompensated heart disease. Protein, or lipotropic substances, may be beneficial in the further progression of the disease and perhaps in the recovery stage.

PROTEIN METABOLISM IN PATIENTS WITH CIRRHOSIS OF THE LIVER*

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The pathogenesis of hepatic cirrhosis is, in some measure, related to protein malnutrition. Contrariwise, established liver disease may affect nutrition, including the metabolism of protein. The purpose of this paper is to present observations relating to protein metabolism in patients with established cirrhosis of the liver. This report is a summary of published investigations concerning this aspect of nutrition in liver disease, and a presentation of data not previously reported. The presentation of data derived primarily from our laboratory is done with a full realization of the valuable contributions of others, many of which have been cited in previous publications.

The data presented were obtained from the study of patients maintained on the Thorndike Metabolic Ward where specialized dietary and nursing services are available. All of the patients studied had advanced cirrhosis of the liver associated with chronic alcoholism and an inadequate dietary intake. They presented obvious clinical and laboratory evidence of parenchymatous liver disease in varying stages of activity and chronicity. In many instances the clinical diagnosis was confirmed by needle biopsy of the liver, or at necropsy.

All of the patients were allowed *ad libitum* ambulation on the ward, none being confined strictly to bed. None presented clinical or laboratory evidence of cardiac or renal disease, nor was any study complicated by vomiting, diarrhea, gastrointestinal bleeding, or febrile illness. Although anorexia may be a prominent symptom of liver disease, this factor does not influence the metabolic data presented since the patients were maintained on constant intakes of food as indicated. The methods of metabolic study, the various analytical procedures, and the clinical laboratory tests involved have been described in previous publications.

Consideration of the effect of well-established cirrhosis of the liver upon the metabolism of protein is presented in the following sequence: (1) the metabolism of dietary protein; (2) the utilization and excretion of amino acids; (3) the untoward consequences of the administration of certain nitrogenous substances; and (4) finally, an evaluation of these data as they apply to the dietary management of patients with this disease.

1. Protein

That digestion and absorption of ingested protein is normal in patients with cirrhosis of the liver is indicated in FIGURE 1, which demonstrates that stool nitrogen excretion is not increased above normal over a range of protein intakes of from 0 to 100 gm. daily. For example, the patients ingesting 75 gm.

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* This investigation was sponsored, in part by the Commission on Liver Disease, Armed Forces Epidemiological Board, with support, in part by grants from the Office of the Surgeon General, United States Army, Washington, D. C., and in part from Merck and Company, Rahway, N. J., to Harvard University.

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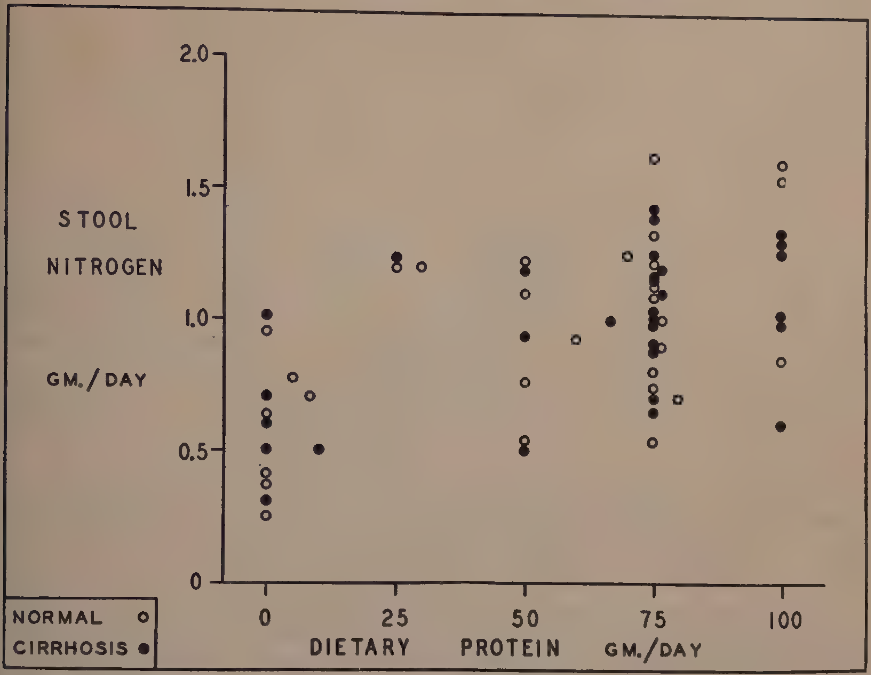

FIGURE 1. Stool nitrogen output in patients with cirrhosis of the liver and in normal subjects at various levels of dietary protein intake.

of protein daily excreted from 0.7 to 1.4 gm. of fecal nitrogen daily, the normal subjects from 0.5 to 1.6 gm. daily, ranges of values which are comparable in the two groups. The analyses of feces for nitrogen were made on from one to five, six-day pools in each patient during which the patient ingested a constant diet. Only the average daily excretion during the entire period of each patient studied is indicated in FIGURE 1. Studies of the metabolic rate (specific dynamic action) and the urinary excretion of nitrogen and of sulfur following the ingestion of protein in large quantities also attest to the usually normal rate of absorption and metabolism of protein in severe cirrhosis.[1]

During studies designed primarily to elucidate the role of dietary factors on the course of cirrhosis,[2, 3] the urinary nitrogen excretion was determined during periods in which patients ingested food providing calories but essentially devoid of protein nitrogen. The minimal urinary nitrogen excreted under these circumstances provided evidence concerning the rate of breakdown of "endogenous" protein. These data are presented in TABLE 1. Three of the patients were given diets containing 0.7 to 2.1 gm. of nitrogen and providing 2500 to 3500 calories daily as carbohydrate and fat. These patients excreted from 2.3 gm. to 4.7 gm. of urinary nitrogen daily. Stool nitrogen excretion was about 0.6 gm. daily. Four other patients, receiving 1600 calories as glucose solution orally and from 0.2 to 0.3 gm. of nitrogen daily, excreted 2.1 to 4.1 gm. of urinary nitrogen daily. These values for urinary nitrogen excretion under conditions of minimal nitrogen intake indicate that the catabolism of

TABLE 1

CIRRHOSIS OF LIVER MINIMAL NITROGEN EXCRETION

Patient	Days of study	Calories daily	Nitrogen gm. per day (average)			
			Intake	Urine	Stool	Balance
J. S.	7	3500*	2.1	4.7	0.5	−2.2
M. P.	11	2500*	0.3	2.7	0.7	−3.1
F. C.	13	2500*	0.7‡	2.3	0.6	−3.1
J. Mc.	10	1600**	0.3***	2.9	0.6†	−3.2
H. C.	4	1600**	0.2***	2.1	0.6†	−2.5
S. C.	5	1600**	0.3***	2.5	0.6†	−2.8
W. S.	5	1600**	0.0	4.1	0.6†	−4.7

* Fat and carbohydrate.
** Glucose.
‡ The nitrogen contained in choline chloride given orally during days 5 to 10 inclusive is included in this value.
*** This nitrogen was contained in choline dihydrogen citrate given orally daily.
† Estimated.

"endogenous" nitrogen was not accelerated. Further, the ingestion of 400 grams of glucose (20 per cent solution in water or in saline), provided sufficient calories to prevent the utilization of significant quantities of nitrogen to meet the energy requirement under the conditions of the study.

The data to follow concern the utilization of dietary protein by patients with cirrhosis of the liver. Although many studies indicate that patients can be maintained in positive nitrogen balance, the question is sometimes raised as to how much protein is necessary to accomplish this object. No information is available to indicate the minimal protein requirement for the maintenance of nitrogen balance. To estimate this requirement and to determine whether or not dietary protein is efficiently utilized, nitrogen balance studies were analyzed. Each patient ingested a constant diet providing from 1600 to 3500 calories. The studies of 16 patients are presented in FIGURE 2, which shows the nitrogen balance of these patients during successive metabolic periods of from six to twelve days each during which the dietary protein intakes were progressively increased, and indicates that the positivity of nitrogen balance is directly related to the protein intake. The observation that positive nitrogen balance can be attained with moderate intakes of dietary protein suggests that these patients are able to utilize dietary protein and that they are protein depleted. All patients ingesting four grams or less of nitrogen daily are in negative nitrogen balance. All patients receiving 12 gm. or more of nitrogen daily are in positive nitrogen balance. Most patients given 8 gm. of nitrogen daily, are in positive nitrogen balance. It might, therefore, be conservatively stated that most patients with cirrhosis uncomplicated by other disease will maintain nitrogen balance if provided with daily diets containing 12 gm. of nitrogen (75 gm. of protein) and adequate calories. These data indicate that even the exceptional patients with the highest requirement for protein would maintain nitrogen equilibrium if given about 9 gm. of nitrogen daily (56 gm. of protein), although most patients require less than this amount.

That protein intakes of 75 gm. daily will maintain positive nitrogen balance in patients with severe liver disease is further indicated by nitrogen balance

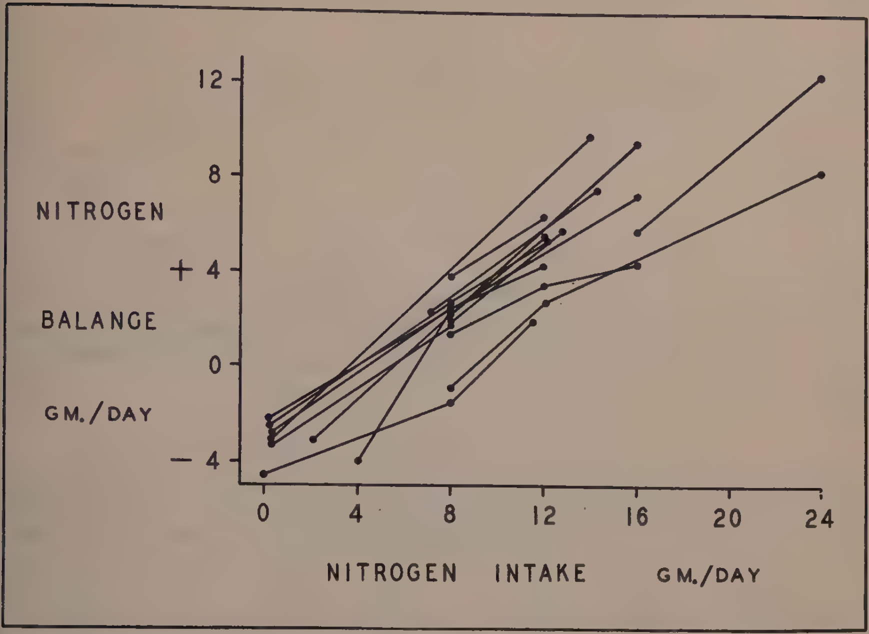

FIGURE 2. Protein requirement of patients with cirrhosis of the liver for maintenance of nitrogen balance.

data obtained by the study of 16 additional patients with ascites who were given diets providing adequate calories and minimal intakes of sodium for study periods of from 15 to 30 days each. A summary of the data obtained from these studies is presented in TABLE 2. Nitrogen balances in this group of patients ranged from +1.6 to +6.8 gm. daily, and averaged +3.8 gm. daily, none being in negative balance under the conditions of study. Thus, the protein requirement of these patients for the maintainance of nitrogen equilibrium or positive nitrogen balance may be greater than for normal individuals but is low enough to insure positive nitrogen balance when 75 gm. (or even 56 gm.) of protein is given daily when adequate calories are also provided. "High" protein intakes were not necessary to achieve a positive balance.

The data presented do not indicate that there is maximally efficient utilization of dietary protein by patients with liver disease. Comparison of these patients to equally undernourished individuals without liver disease with regard to the efficiency of utilization of protein awaits some means of adequately evaluating an individual's state of protein nutrition. Our clinical impression has been that, nutritionally, it is more difficult to rehabilitate patients with chronic cirrhosis of the liver than patients who are undernourished due to starvation. The problem, however, has been approached in another manner. Selected patients with evidence of severe active liver disease were maintained on constants diets, and urinary nitrogen excretion was followed for 40 to 50 days during which progressive improvement, as judged clinically and by laboratory tests, was manifest. A progressive decrease in the urinary excre-

TABLE 2

NITROGEN BALANCE, SIXTEEN PATIENTS WITH CIRRHOSIS WITH ASCITES; 15–30 DAYS STUDY PER PATIENT; 2500–3500 CALORIES PER DAY

	Nitrogen gm. per day			
	Diet	Urine	Stool	Balance
Range	10.8–13.8	4.5–8.9	1.0–1.5	+1.6–6.8
Average	12.3	7.5	1.0	+3.8

tion of nitrogen was evident. The data obtained from the study of two patients in this manner are presented in FIGURE 3. These studies suggest some defect in the utilization of dietary protein in the presence of active disease and a more efficient utilization with improvement.

2. *Amino Acids*

The plasma concentration and the daily urinary excretion of alpha amino nitrogen were normal (TABLE 3), and the quantity of each of the ten "essential" amino acids excreted in the urine by patients with cirrhosis of the liver have been found not to differ markedly from that observed in normal individuals maintained on comparable diets.[4, 5] The most marked deviations from the normal noted were an increased excretion of methionine and of tryptophan and a decreased excretion of isoleucine. The increased excretion of these two essential amino acids was not considered to be nutritionally limiting nor to alter the patients' requirements for them. This is substantiated by the failure of dietary supplements of methionine (and choline) to enhance nitrogen balance in patients with hepatic cirrhosis.[6] However, the failure to observe gross deviations from the normal pattern of urinary amino acid excretion does not imply that the intermediary metabolism of each of the amino acids is entirely normal.

In order to define further any abnormality in the urinary excretion of amino acids by patients with cirrhosis two additional methods of study were employed.[5] One of these, designed to determine the effect of increases in dietary protein intake upon the urinary excretion of amino acids, demonstrated that increases in protein intake did not result in the urinary wastage of amino acids. During the other, the effect of the severity of the liver disease upon the urinary excretion of the ten "essential" amino acids was noted by observing the alterations in their urinary excretion which occurred concomitant with clinical improvement in patients provided with adequate and constant diets. Progressive decreases in the urinary excretion of these amino acids were noted, suggesting a more efficient utilization of amino acids with improved liver function. The conclusion drawn was that the activity of the patient's disease influenced the urinary excretion of these amino acids more than did alterations in the dietary protein intake. These data, indicating a decreasing urinary excretion of amino acids with clinical improvement, and the studies mentioned above, which indicate a decreasing urinary excretion of nitrogen under similar conditions of study, suggest the presence in patients with severe liver disease of an

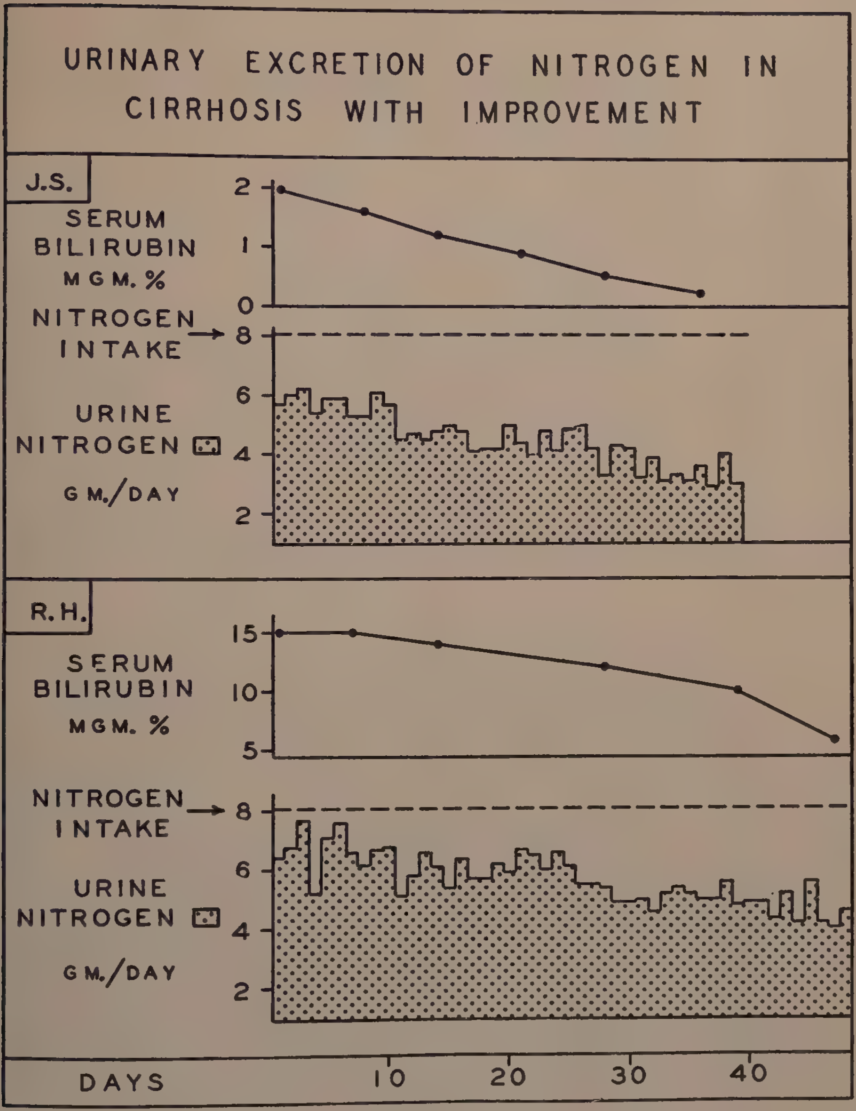

Figure 3.

abnormality in the utilization of dietary protein. Whether these findings are unique to patients with liver disease or also occur in patients recovering from various other illnesses has not been established. It should be noted that only a small portion of the observed decreases in urinary nitrogen excretion can be accounted for by decreases in "essential" amino acid nitrogen. Partitioning of urinary nitrogen under these conditions of study was not done.

Patients with liver disease whose intakes of food may be limited by anorexia

TABLE 3

PLASMA AND URINE ALPHA AMINO NITROGEN

	Patients with cirrhosis	Normal subjects
Plasma, mgm./100 ml.		
No. pts.	12	35
Range	2.7–4.6	2.9–4.5
Average	3.9	3.7
Urine, mgm./24 hr.		
No. pts.	15	10
Range	84–258	118–199
Average	171	152

TABLE 4

PLASMA ALPHA AMINO NITROGEN (MG. PER 100 ML.) FOLLOWING INTRAVENOUS INFUSION OF 300 ML. 15% PROTEIN HYDROLYSATE

	Before infusion	After infusion	
		1 hr.	4 hr.
Patients with cirrhosis (8)			
Range	2.7–4.3	6.3–12.6	3.4–8.2
Mean	3.7	8.3	5.5
Normal subjects (5)			
Range	3.5–4.5	5.0–9.6	4.2–4.9
Mean	4.0	7.5	4.5

or other digestive disturbances may require the provision of nutrients by parenteral feeding. Since the patients with damaged livers might not metabolize amino acids normally when given parenterally, the advisability of administering amino acids to these patients has been questioned. For these reasons, studies were undertaken to evaluate the tolerance, excretion, and therapeutic value of the intravenous administration of solutions of amino acids or protein hydrolysates to patients with cirrhosis of the liver. Their tolerance to the rapid intravenous infusion of a protein hydrolysate (300 cc., 15 per cent solution) was not grossly abnormal (TABLE 4). The clearance of amino acids from the blood by the patients was only slightly delayed as compared to normal individuals.[7] Furthermore, in one study, a progressive rise in serum alpha amino nitrogen concentration did not occur in a patient receiving 100 gm. of amino acids intravenously daily for 18 days.[8]

Further evidence that intravenously administered amino acids are metabolized comes from the nitrogen balance studies over 2 to 4 weeks of four patients with active cirrhosis of the liver, given a solution of amino acids intravenously daily as the sole source of nitrogen, while they were given a diet essentially devoid of protein and furnishing adequate calories.[8] Each of these patients achieved positive nitrogen balance when provided with adequate quantities of amino acids. Azotemia or hyperaminoacidemia did not occur. In addition, improvement in the liver disease, as evidenced clinically and by laboratory tests of liver functions, was observed during this period. The pattern of the infused amino acids which were excreted into the urine by the

TABLE 5

PATTERN OF URINARY AMINO ACIDS EXCRETED FOLLOWING AMINO ACID INFUSIONS TO NORMAL SUBJECTS AND TO PATIENTS WITH LIVER DISEASE

Amino acid	Per cent of total of ten "essential" amino acids		
	Given	Excreted	
		Normals (average 8 subjects)	Cirrhosis (average 4 patients)
Arginine	4.2	1.8	0.7
Histidine	6.6	16.1	15.6
Isoleucine	14.1	4.7	7.8
Leucine	28.1	14.0	17.5
Lysine	17.3	20.6	21.5
Methionine	6.7	8.1	7.2
Phenylalanine	6.9	9.2	6.9
Threonine	3.0	13.8	8.6
Tryptophan	1.5	2.2	2.3
Valine	11.6	9.5	11.9

patients differed little from that noted in normal individuals given similar infusions, as illustrated in TABLE 5.

3. *Other Nitrogenous Substances*

Interest in the metabolism of nitrogenous substances was stimulated by the observation that some patients with cirrhosis of the liver, given an ammonium containing cation exchange resin as a diuretic agent, developed a syndrome indistinguishable from impending hepatic coma.[9] This syndrome typically consists of mental disturbances (*e.g.*, drowsiness, confusion, disorientation, and inappropriate behavior), a characteristic coarse irregular flexion-extension tremor best elicited in the out-stretched hands, and an electroencephalographic pattern with bilaterally synchronous slow waves of about two per second activity. In a subsequent study,[10] it was shown that this syndrome simulating the neurological findings seen in spontaneously developing impending hepatic coma also occurred in certain patients with cirrhosis given other nitrogenous substances orally, including ammonium chloride, diammonium citrate, urea, and large amounts of dietary protein. The precipitation of the untoward reactions did not correlate with serial measurements of serum pH, and sodium, potassium, and carbon dioxide concentrations, or with blood nonprotein nitrogen levels. Although "blood ammonia" levels were frequently elevated in these patients during these reactions, the correlation was not consistent enough to establish a direct relation between the two. "Blood ammonia" concentrations were also frequently elevated in patients developing hepatic coma spontaneously, but not inevitably so.[11, 12] In spite of this lack of correlation, the fact that all of the substances which produced the untoward reaction provided ammonia (ammonia may have been produced from urea and dietary protein by the action of intestinal bacteria), make it tempting to speculate that ammonia may have given rise to the reaction either directly or by causing the formation of some other toxic substance, such as an amine. It is also possible that patients with liver disease are not able to remove ammonia adequately from the blood, due, for example, to possible defects in the intermediary metabolism of glutamic acid.

Thus, although the mechanism of the reaction noted in the patients with cirrhosis of the liver given the nitrogenous substances is not clear, the studies suggest that the administration of these materials may be harmful, and also offer additional evidence that a defect in nitrogen metabolism exists.

All patients given nitrogenous substances did not develop the untoward reaction and, although all patients studied presented evidence of severe liver disease, whether or not a patient had a reaction did not relate to liver function as measured by the usual tests. For example, only two of six patients in whom the dietary protein intake was increased to 150 gm. daily developed untoward symptoms.[10] Subsequent to this study, three additional selected patients have been observed who displayed neurological signs attributable to increases in dietary protein intake. Even this incidence of reactions following dietary protein is significant, since large quantities of dietary protein are frequently recommended in treatment. On the basis of the observations cited, the advisability of providing large quantities of nitrogenous substances including dietary protein to patients with severe liver disease, especially those with hepatic coma, may be questioned.

Summary

Data have been presented which demonstrate that the absorption of nitrogen was normal in 28 patients with cirrhosis of the liver. Studies of urinary nitrogen excretion in seven patients given 1600 to 3500 calories and diets providing minimal intakes of nitrogen indicate that "endogenous" protein was not catabolized at an accelerated rate. Each of 32 patients studied demonstrated positive nitrogen balance when provided with 75 gm. of protein daily. In fact, most of these patients maintained positive nitrogen balance, when provided with 50 gm. of protein daily in a calorically adequate diet. Four additional patients also maintained positive nitrogen balance when given adequate quantities of amino acid solutions intravenously as a sole source of nitrogen. "High" protein intakes were not necessary for patients with well-established cirrhosis of the liver to achieve positive nitrogen balance.

The urinary excretion of "essential" amino acids by patients with cirrhosis did not deviate grossly in pattern or in quantity from that observed in normal individuals given comparable diets or intravenous infusions of protein hydrolysate. The urinary excretion of amino acids was affected more by the severity of the liver disease than by the dietary protein intake. Following the rapid intravenous infusion of amino acid solution, their clearance from the blood was only slightly delayed in the patients. These data do not imply that the intermediary metabolism of each of the amino acids or of the nonessential amino acids is normal in patients with liver disease.

A defect in the utilization of ingested protein is suggested by the somewhat greater protein requirement for the maintenance of nitrogen balance of some of the patients than that for normal individuals, by the decreases in urinary nitrogen and amino acid excretion observed with clinical improvement and,

finally, by the untoward reactions noted following the administration of nitrogenous substances including dietary protein.

The studies presented do not indicate to what extent the dietary protein intake is related to the completeness or rapidity of recovery of patients with liver disease. However, it is apparent that patients with cirrhosis maintain positive nitrogen balance when given moderate quantities of protein, and that large amounts of nitrogen may be harmful. A diet providing an "adequate" or "normal" protein intake may be best suited for the treatment of patients with this disease.

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TREATMENT OF INFANTILE CIRRHOSIS OF THE LIVER WITH ANTIBIOTICS

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Infantile cirrhosis of the liver is a peculiar condition found in Indian children. The disease commences insidiously between the ages of 6 and 18 months, during which period the liver begins to enlarge gradually up to a size of four or five fingers' breadth with a comparatively small increase in the size of the spleen. In the last stages of the disease, there may or may not be jaundice, but ascites is an invariable sequence. At the final stages, death is due to cholemia or hemorrhage.

In my previous publication (1941), it was pointed out that the pathology of infantile cirrhosis is that of Laennec's (portal) type and that the etiology falls into two stages. It is my belief that, in the early stage, there is degeneration of the liver, due to dietetic error (feeding children entirely on cows' milk) and that, subsequently, formation of cirrhosis occurs, due to infection of the liver by *Escherichia coli*. Recent work on cirrhosis of the liver by various other authors has discussed the view that lack of vitamin B factors, especially choline in food, may be involved in producing cirrhosis of the liver. Bicknell and Prescott have said that milk is not a good source of choline (1946).*

In my previous paper (1951) on the treatment of infantile cirrhosis of the liver, it was shown that oral administration of choline and injections of Streptomycin have proved successful. The object of the present paper is to indicate briefly a further note on Streptomycin and the effects of other antibiotics in the treatment of cirrhosis of liver.

Streptomycin. Many more cases have been treated with this drug since 1951 and the results have been uniformly good. In fact, this has become the routine treatment for infantile cirrhosis cases in this part of India. The usual course is 4 to 5 gm., injected in daily doses of ¼ to ½ gm., continued over a period of 15 to 20 days.

Aureomycin. Four cases were treated with this antibiotic. Aureomycin spersoids were used throughout. One teaspoonful three times a day, giving a total quantity of 150 mgm. of Aureomycin HCl was the routine dose for early cases; and advanced cases received 250 mgm. or more per day. Diarrhea was the usual complication, and the drug had to be stopped in the middle of the treatment for one, two, or even three days, until the diarrhea was controlled. All the children died. Therefore, this drug has not proved useful in the treatment of infantile cirrhosis of the liver.

Terramycin. The next antibiotic being tried is Terramycin. This is given in the form of drops, the powder being dissolved in the solvent supplied by the manufacturers. A daily dose of 600 mgm. is administered in three divided doses. Six cases have been tried so far, for the last six months. Marvelous results have been observed. In the case of four children with an early stage of liver enlargement (about 2 fingers), the liver came to normal size by the time 2 gm. of the medicine were completed. Tonics containing choline, methionine, and vitamin B complex were given simultaneously as adjuvants. Even in two other cases which were advanced with jaundice and ascites the results were encouraging. Jaundice disappeared in the course of one week and the enlarged liver was reduced to half the size. These two children were given a total quantity of 6 gm. with daily doses of 600 mgm. Terramycin may, perhaps, prove to be the drug of choice so far as this disease is concerned. These two children who would have died otherwise are still alive, and the liver is reduced to two fingers' breadth. Further progress is being watched carefully.

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* Work is being carried out here in collaboration with Sri M. Bhimsena Rao, a Fellow of the Raptakos Medical Research Board (Bombay) and Doctor K. P. Basu at the Indian Dairy Research Institute, Bangalore, on the choline contents of various milks, human as well as other animals, and also of the mothers of cirrhotic children. The results will be published in course of time.

Remarks

I am aware of the fact that the number of cases treated is very small to draw any definite conclusions. In accordance with my theory that *E. coli* is responsible for bringing about the cirrhotic changes in the liver, I tried all the various antibiotics that have been proved to act against this bacteria. During the course of these investigations, Terramycin has proved to be so very efficacious that I feel there is justification in giving publicity to these findings, however small the number of cases may be. It is to be particularly emphasized that great difficulty is experienced in getting a larger number of cases for treatment, as the parents refuse to leave their children in the hospital for two to three months continuously, for economic or other reasons. At the same time, it is not desireable to treat them as out patients, because one cannot be sure whether the treatment would be continued as planned or that the parents might not introduce other drugs in their anxiety to bring about a cure quickly, thus vitiating the results. It is hoped that a combination of the antibiotics, Streptomycin and Terramycin, may prove to be specific in the cure of infantile cirrhosis and, as such, a trial should be carefully conducted and reported.

Summary

In this series of cases, in addition to Streptomycin, two new antibiotics, Aureomycin and Terramycin, have been tried in cases of infantile cirrhosis of the liver. Terramycin has been found to be very efficacious in curing the condition.

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METABOLIC AND NUTRITIONAL PATTERNS IN ALCOHOLISM

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The reasons for considering the nutritional and metabolic patterns in human alcohol addicts as differentiated from those of nonalcoholic persons are two; the one based on clinical and the other on experimental viewpoints. The clinical reason is that certain deficiency diseases appear with significantly greater frequency in alcohol addicts than in nonalcoholics. These are liver cirrhosis, polineuropathy and other forms of thiamin deficiency, and pellagra. The experimental reason is that it has been observed in rats and mice that when their diet lacks certain B complex vitamins they increase their voluntary ethyl alcohol intake, thus showing that nutritional factors can alter the appetite for alcohol.

In human beings, the nutritional conditions depend, on the one hand, on the characteristics of the appetite, and on the other, on the ability to satisfy it. I use the term "appetite" not in the restricted sense of the desire to ingest food in general, but to eat certain foods.

Human appetite has a very complicated mechanism, and it differs in each person. Fundamentally, differences in the appetite are the result of genetic constitution, as well as of characteristics acquired by experience, education, and pathological events. In their daily fluctuation, they are the result of physiological changes and pharmacological actions.

Thus, if a group of persons shows a nutritional pattern, it means that there are, in the constituents of the group, certain similitudes in the factors affecting nutritional conditions; namely, genetic constitution, experience, education, pathological events, and habitual pharmacological actions affecting the appetite as well as the economic possibilities of satisfying it.

It is easy to understand, for instance, why a specific nutritional pattern may exist in the social classes of low economic level in certain countries in which the common factor of limited availability of foods is present. Obviously, examples of this nature can be multiplied.

In the case that concerns us now, however, in which we assume the presence of a nutritional pattern in a group, not as the result of a dietary survey, but because of the frequency of deprivation diseases, the problem increases its difficulties because of the appearance of new variables. In fact, the individual vatiations are now playing a new role since, with the same deficient diet, different individuals show differences in the development of pathological consequences. Such individual differences are produced particularly by genetic constitution and also by the occurrence of pathological conditions.

All the preceding considerations force us to conclude that, if thiamin deprivation symptoms are frequent in alcohol addicts, it means that the addiction leads the patient to consume a diet low in thiamin or that a relationship of some other nature exists between addiction and thiamin deprivation. In fact, it is possible that alcohol addicts are more sensitive to thiamin deprivation or that thiamin-deprived persons are more likely to become alcohol addicts. *Mutatis mutandi*, the same can be said concerning liver cirrhosis and low protein diet, as well as in the case of pellagra and nicotinic acid deficiency.

Let us now consider each of these points. There is no doubt that, during their so-called "benders," alcohol addicts reduce their food intake to undernutrition levels, and this may be the cause of deficiency diseases; but, on the other hand, we have to consider that undernutrition is frequent enough in certain populations so that a more even distribution of these diseases may be expected between alcoholics and nonalcoholics. Thus, it is not wise to conclude that alcoholism is merely a cause of undernutrition and thus to close the door to research on other possible relationships between nutritional deficiencies and alcoholism.

Obviously one question must be posed first; namely, does alcohol ingestion increase the sensitivity of individuals to these deficiency diseases?

Concerning thiamin, it has been established by Lowry, Sebrell, Daft, and Ashburn[6] that alcohol ingestion prolongs the survival time of rats fed on a diet lacking thiamin, and Westerfeld and Doisy[22] reported similar results in pigeons. This means that alcohol does not increase the sensitivity to thiamin deficiency but, on the contrary, decreases it. There is no reason to assume that things occur differently in humans. In fact, Butler and Sarett[4] reported that alcohol ingestion increases the excretion of thiamin in humans, meaning that it produces a sparing effect in thiamin utilization.

Concerning the influence of alcohol ingestion on the onset of experimental liver cirrhosis, the results obtained by different workers do not agree, and it is, therefore, too soon to review this problem. In any case, it has not been proved that alcohol enhances the evolution of the experimental disease. In humans it has been observed by Patek and Post[13] that patients with liver cirrhosis and with a history of alcoholism, improved their condition when they received a convenient diet, without cutting down alcohol intake.

I am not aware of any experimental work concerning the effect of alcohol intake on the onset of nicotinic acid deficiency. In humans, Butler and Sarett[4] report an increased excretion of nicotinic acid after alcohol intake. They have interpreted this as the result of a sparing action of the alcohol in the nicotinic utilization.

Thus, it seems clear that if alcohol addicts have an increased sensitivity to the development of these deficiency diseases, it is not the direct effect of the alcohol ingested.

This leads us to think that the observed relationship may be of a different kind and, obviously, it is necessary to test whether or not there are conditions that, at the same time, favor both the development of alcohol addiction and the above-mentioned diseases.

There is no doubt that the psychological factors which play a role in the pathogenesis of alcohol addiction are similar to those which induce other forms of mental trouble. It is, therefore, necessary to assume that there are personal conditions favoring the influence of these psychological factors toward alcohol addiction.

It is now clear why it is important to review the experimental facts that convince us that other kinds of relationships can exist between nutritional conditions and habitual drinking of alcohol.

Richter and co-workers, in papers appearing between 1937 and 1941, reported that when normal rats are free to choose between foods, they eat a balanced diet[18] and that they adapt the diet to the needs changed by experimental endocrine disturbances[14, 16, 19] or by vitamin deficiencies.[17] Richter and Campbell,[15] on the other hand, reported that, under free choice, rats prefer a weak alcohol solution to tap water. Mardones and Onfray, in 1942,[8] having in mind the idea that a disturbance of the carbohydrate metabolism between C_3 and C_2 compounds could increase the desire for alcohol, studied, with rats, the influence of a diet deprived of thiamin on the voluntary alcohol intake. Their findings showed that, in these circumstances, rats significantly increased the alcohol intake. If these rats received a thiamin supplement, they improved their weight and general appearance but they maintained the high-level alcohol intake. If, however, they received a supplement of liver, meat, wheat germ, untreated yeast, the voluntary alcohol intake rapidly reached the normal level. Since the diet lacking thiamin contained autoclaved yeast, we claimed the presence of a new thermolabile factor of the B complex which we designated factor N. Subsequently my associates and I[10] reported that, if the supplement of thiamin was maintained for some weeks, the rats decreased the voluntary alcohol intake which reached the normal level in about twelve weeks; and that if normal rats were fed on a diet containing autoclaved yeast plus thiamin, the voluntary alcohol intake was maintained at the normal level. On the other hand, rats fed a diet containing only ten pure vitamins of the B complex and showing a high-level alcohol intake, decreased the alcohol intake in a similar way after supplementation of yeast, regardless of whether the yeast was autoclaved or not. The thermostable factor present in yeast and liver responsible for the decrease of the alcohol intake in these conditions has been called factor N_1. Actually, these findings show that factor N is composed of thiamin (thermolabile), and N_1 (thermostable).

In the meantime, Brady and Westerfeld[3] reported new facts along this line. These authors confirmed that rats fed on a diet deprived of all the elements of the B complex rapidly increased the voluntary alcohol intake, but they observed that these rats decreased it only temporarily after each continuous supplement of vitamins. On the other hand, they reported that the same diet that prevented the increase of the alcohol intake is unable to maintain it at a low level in rats that have reached a sizable intake. The authors suggested, then, that this fact means that "nonnutritional factors complicate the picture."

The facts reported by Brady and Westerfeld were explained later on. Beerstecher and co-workers[1] showed that a single deprivation of riboflavin, pyridoxine, or pantothenate also increased the voluntary alcohol intake of rats and that it dropped suddenly when the specific deprivation was corrected. This fact led to the interpretation that the transitory drop observed by Brady and Westerfeld was the result of partial satisfaction of the deficiencies that produced the increase of the alcohol intake. On the other hand, we have reported[11] that, if a sufficient amount of yeast supplement is given to rats with high-level alcohol intake, the decrease of the voluntary intake was permanent. Thus, the second fact reported by Brady and Westerfeld is presumably the result of the preventive dose of the new factor being lower than the curative one.

All these findings support the idea that liver and yeast contain a thermostable factor as yet unknown, lack of which in the diet induces an increase in the voluntary alcohol intake of rats.

Recently[9] we have studied the influence of synthetic alpha lipoic or thioctic acid in the alcohol intake of rats fed a diet deprived of factor N_1, and we have observed that, in some rats, this substance significantly decreases the alcohol intake but that, in the same rats, a liver supplement can induce a further decrease and that, in rats in which alpha lipoic acid did not modify the alcohol intake, liver supplement decreased it. If we consider as factor N_1 any thermostable substance present in liver or yeast that can decrease the alcohol intake of rats fed a diet containing the known vitamins of the B complex, we are obliged to state that alpha lipoic or thioctic acid is one of them.

Summarizing the influence of deprived diets on the alcohol intake of rats, it can be said that deficiencies of thiamin, riboflavin, pyridoxin, pantothenate, alpha lipoic acid, and other factors as yet unknown (N_1), will induce an increase of the voluntary alcohol intake in rats.

Williams, Berry, and Beerstecher,[23] at the same time that they confirmed the influence of lack of vitamins of the B complex on the voluntary alcohol intake, reported the presence of strain differences in the responses of rats and mice as well as individual variation in the same strain. Mardones, Segovia, and Hederra[7] also reported the same individual fluctuations in the strain of white rats raised in our laboratory fed either the stock ration or the deprived diet. In our colony the frequency distribution curve was hyperbolic with highest frequency in the low alcohol consumption. The same authors reported later on[12] that the coefficient of heredity of the alcohol intake of rats fed a diet lacking factor N_1 was $r = +0.416$ and $t = 8.72$ in the third to seventh generation of inbreeding, meaning that it is extremely improbable that this correlation was the result of chance.

All these facts show that it is highly probable that the level of voluntary alcohol intake of normally nourished rats as well as of rats fed a deprived diet, is determined by genetic factors. It also seems possible that this hereditarily transmitted condition can be expressed in a metabolic pattern leading to an excessive ingestion of alcohol. This working hypothesis has not been tested as yet in experimental animals, but something has been done in human alcohol addicts. Beerstecher and co-workers[2] have observed some differences in biochemical conditions between alcohol addicts and normal persons. The statistical significance of these differences is questionable because of the small number of individuals in both groups. Differences were observed in citrate, hippuric acid, uric acid and citrulline in urine, sodium content of saliva, and magnesium level in blood. No biochemical reason has been postulated to explain the relation between these findings and the excessive alcohol intake.

Recently Varela, Penna, Alcaino, Johnson, and Mardones[21] have initiated a study of the carbohydrate metabolism in human alcohol addicts with the idea that a disturbance in it could explain why the energy of the alcohol is more available to alcoholics than that of the carbohydrates. Their findings, so far, show that the glucose tolerance did not differ in the group of alcohol addicts from that of social drinkers and abstainers, but that some differences between the two groups in the blood pyruvate curve and in the blood acetaldehyde curve, after glucose ingestion, are significant at the 2 per cent level. These results cannot yet establish the presence of a metabolic disturbance specific for alcohol addiction, but it encourages new studies along the same line.

After this paper was prepared, I became aware of some new facts on which I wish to comment briefly as follows:

Recently Lester, Greenberg, Smith, and Wu[5] reported a finding that interferes with the interpretation of the voluntary alcohol intake in rats. They observed that, if rats showing high-level alcohol intake were allowed to drink sucrose or saccharin solutions, they decreased significantly the alcohol intake. It is not easy to interpret this fact since those rats had free access to a diet containing sucrose, which means that their appetite for this sugar differed according as it was in solution or in crystalline form. On the other hand, the interpretation is complicated by the circumstances that the experimental rats used showed a very high-level alcohol intake; that the diet was low in carbohydrate; and that the rats were not actually depleted. In any case, however, these results would necessitate study of the effect of nutritional imbalance on the selection between an alcohol solution on the one hand, and a sucrose solution on the other, instead of a dry diet containing crystalline sucrose.

Two other facts have been reported that are related to the problem under discussion. The first one was published by T. B. Sirnes[20] of the Department of Pharmacology of the University of Oslo. He observed that rats in which liver cirrhosis was induced by treatment with carbon tetrachloride increased significantly their voluntary alcohol intake, and that this increase is the consequence of the cirrhosis induced and not of other pharmacological effects of carbon tetrachloride. This shows that a liver lesion can induce high-level alcohol intake.

The other fact is reported in this monograph by Olson and Dinning (see page 889). They observed that, in the liver of rats with dietary necrotic liver degeneration, there is an important depression of the pyruvate oxidase.

All the facts analyzed in this talk induce us to believe that voluntary alcohol intake is increased when a difficulty in the utilization of the energy of carbohydrates or fat is present, and that this difficulty does not interfere with the utilization of the energy of the alcohol. They also induce us to suspect that, in some cases, the excessive ingestion of alcohol is not the cause but the consequence of nutritional or metabolic difficulties.

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THE GENETOTROPHIC CONCEPT—NUTRITIONAL DEFICIENCIES AND ALCOHOLISM

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A full appreciation of the general and far-reaching significance of the genetotrophic concept[1, 2] requires that we recast, if not revolutionize, our ideas regarding how we should use statistical methods in our attempts to solve biological and human problems.

The revolutionary point of view which enters into this concept is in line with the current opinion of geneticists that every genotype determines its "norm of reaction" to the environment[3] and that no species can adequately be described in terms of a type specimen. It is also in accord with the idea of partial genetic blocks[4, 5] which recent evidence has indicated to be the rule rather than the exception.[6]

In the areas of physiology, psychology, biochemistry, and medicine, as well as in philosophy and the social sciences, we have built into our thinking the concept of *the normal man* whom we regard as of paramount importance. We have set it as our supreme task to understand how this hypothetical creature functions. In the field of animal biology, the rat, the guinea pig, or the salamander, etc., become the center of our attention. This is the view which is incompatible with the genetotrophic concept.

Biological variability is recognized by the biological fraternity as a more or less necessary evil. When it is extreme, it makes very difficult the establishment of norms. However, by proper statistical treatment, its effects are smoothed out and we are, as it were, back on the right track again. The tacit assumption is made that the normal man is normal through and through, and that abnormalities are to be observed only in those who belong in the category of the "abnormals." This assumption is manifestly false, as will be made clear in our discussion. Nevertheless, it is commonly accepted and underlies the firmly intrenched idea of the normal man (or rat or guinea pig) which appears to be a guiding concept for most monographs, advanced books, and research publications in all of the areas of biological science.

This attitude, which centers its attention on those supposed individuals whose attributes always lie within the normal range, is based in part upon another idea which is firmly intrenched in much biological thinking, namely that, on the microscopic level, "the cell" is the center of our interest. If, we say, we can ascertain how "the cell" is constructed and how it works, we will have gone a long way toward understanding biology.

The fundamental reason why we construct "the normal man" in our minds is so we can develop *generalizations*, which in a sense are the essence of science. Scientific progress in the area of biology as well as in other areas may be measured in terms of the number and scope of valid generalizations which exist; the more far-reaching the generalizations, the more advanced the science.

It is my broad thesis, however, that biological variability in the human species is so very great and so significant in the lives of all of us that, unless we *center attention on variability*, we shall find ourselves quite unable to apply biology to the solution of human problems. Generalizations are necessary and inevitable, but those which are made without regard for human variability are very likely to be premature and completely misleading. Leo Loeb,[7] who has devoted a lifetime of study to variability, has said that individuality is most highly developed in the human species, and this observation alone should make us alert to its possible implications.

Let us look hastily at a number of human problems which naturally fall in the hitherto undeveloped field of *applied human biology*. Consider sex crimes for example. Can one be at all sure that "the normal human being" commits these, and that *deviates* in the area of sex physiology are not responsible? If we admit that this is an area where deviation may be important, how about the broader problem of sex adjustments in which all human beings are concerned? Can anyone say *a priori* that these adjustments characteristically involve ' the normal human" and that the enormous variability which Kinsey's report brought to light (much of which is probably basically inborn) has nothing to do with these adjustments? Marriage and divorce, and hence our whole social structure, have deep biological roots, and it seems probable that the enormous variability in sex urges, inclinations, and predispositions are of paramount importance and that the very deviations (which may be found in any of us) merit serious study instead of being smoothed away by statistical techniques.

I should like to interpose here another reason why "man" or "the normal human being" has in the past been so much the center of interest. We should like to think that, at the beginning of life, we have an equal start, and that we are the makers of our own destinies. If many of our problems arise out of our hereditarily controlled variation, we reach an "apparently gloomy conclusion," as Professor Dunn[8] puts it, "toward which all results of heredity seem to be driving us," namely, that our shortcomings and hence our difficulties are foreordained and hence irremediable. This conclusion is by no means a valid one, and the genetotrophic concept points the way, very significantly, as we shall see later, toward its demolition or at least its radical modification.

Consider next, if you will, the broad problem of health as a field of applied human biology. How many so-called metabolic diseases have their roots in inborn metabolic peculiarities? We know, for example, that diabetes does and, on the basis of present evidence, it seems probable that other metabolic diseases such as arthritis, gout, obesity, hyperinsulinism, *etc.*, also do. Such a question as "Why does *man* (the typical human being, who is supposedly normal through and through) become diabetic?" is probably a completely misleading and unanswerable question.

If we concede that inborn variability may be important in connection with metabolic diseases, what shall we say about infective diseases? Professor Gowen of Iowa State College has recently shown,[9] by combining a genetic study of mice with a study of their responses when challenged with tuberculosis germs of varying virulence, that the problem is three-dimensional, as is shown in FIGURE 1.

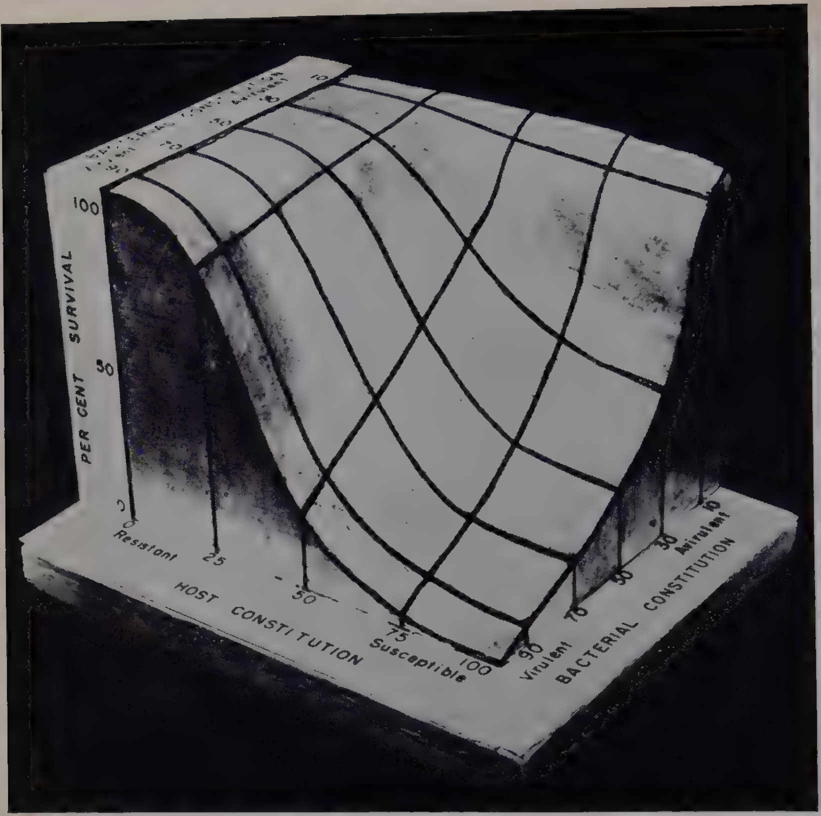

FIGURE 1

It would be difficult to controvert the idea that *all* infective diseases are three-dimensional in the same way.

There are numerous other diseases not classified as metabolic or infective, for which the etiology has been for decades completely unknown or at least obscure. Among these are cancer, allergies, mental disease, alcoholism, dope addiction, and many so-called degenerative diseases.

In all of these diseases, a common fact may be observed. The diseases attack only *certain individuals* while others in the same environment escape completely. One member of a community may have cancer at a relatively early age. Another, who has the same occupation, eats the same food, breathes the same air, drinks the same water, lives in a similar house, is exposed to the same sunshine and weather, may live to be 90 years of age with no threat of the disease. A similar statement applies in the case of allergies. Some people are unfortunate; others are fortunate. One member of the community may develop mental disease, while his fellows, who have perhaps just as many harassments and frustrations, remain well-balanced. Two individuals in the same environment can drink alcoholic liquors to the same extent over a period of years. One may, however, become an alcoholic, an uncontrolled drinker, while the other may remain a heavy but well-controlled drinker. When different individuals under hospital conditions are repeatedly given morphine, most of them escape aftereffects, but some, for no known reason, become addicts. Heart disease is a type of degenerative disease which attacks certain individuals and not others, and there is apparently no environmental influence which determines who will be the victim.

What hypothesis regarding these diseases can match for importance the one which postulates that, in every case, the individual's predispositions rest upon biological deviations from the respective norms? "Why is the 'normal human being' subject to allergy? Why does the 'normal human being' become an alcoholic? Why does the 'normal human being' have heart disease?" These, from the standpoint of our discussion, are foolish and misleading questions. Far more in order are the questions: "What deviations do we find in those subject to cancer? What metabolic peculiarities make one subject to allergy? What is different about certain persons which makes them become alcoholics? What kinds of deviations do we find accompanying specific types of heart disease?"

It seems probable that learning about ' normal" characteristics is only a first step. We must learn more and more about deviations, and in order to do this, we must give them serious and attentive study. We must not eliminate them by statistical treatment. Instead we must search for them and discover their nature. We must scrutinize them. We must find out about their frequencies and be continuously aware of their existence and their possible influence. I believe convincing evidence is actually at hand which shows that, mathematically and scientifically speaking, no such thing exists as a human being who is normal through and through. An individual may be "normal" with respect to one item in his existence but not for all the items that enter into his makeup. This is illustrated in part by some data[10] I was fortunate enough to find in the biochemical literature, which pertained to the blood lipid levels exhibited by 67 "normal" individuals. In this article, unlike most, complete individual data are given.

FIGURES 2, 3, and 4 are distribution diagrams published by Page, Kirk, Lewis, Thompson, and Van Slyke, having to do with blood levels of (1) lipid phosphorus, (2) free cholesterol, and (3) lipid amino nitrogen. From FIGURE 2 and the accompanying tabulated data in the original article, it is evident that four individuals lie below the normal range (at the 95 per cent level): numbers 5, 18, 29, 67; and one individual, number 63, lies above this range. From FIGURE 3 and the accompanying data, it is clear that there are two individuals below the normal, 51, 56; and three above, 24, 42, 61. But these are not the same individuals that appeared abnormal in FIGURE 2. They constitute a completely new set of "abnormals." From FIGURE 4 and the accompanying data, one individual, number 13, is below the normal range, and two, numbers 35 and 51, are above it. Here appears another new set of "abnormals" entirely different from the others.

It does not take very much imagination to conclude that, if these 67 indi-

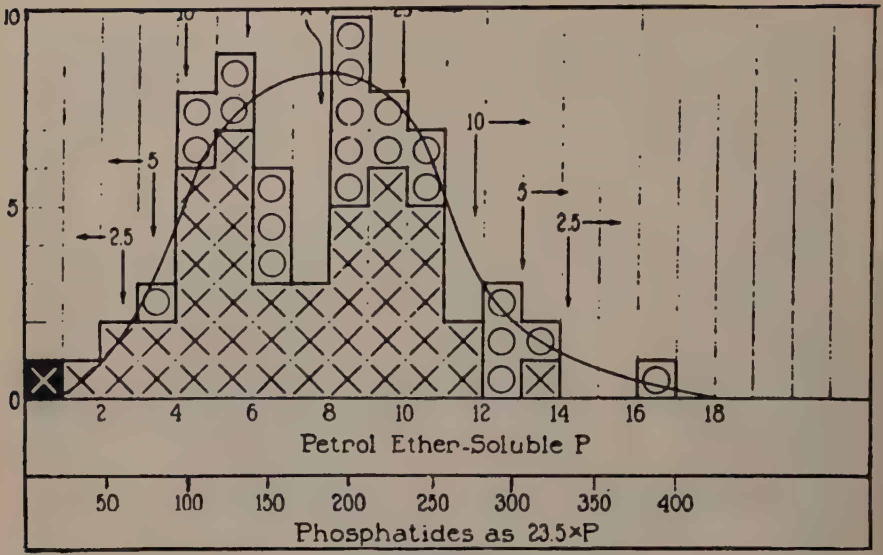

FIGURE 2

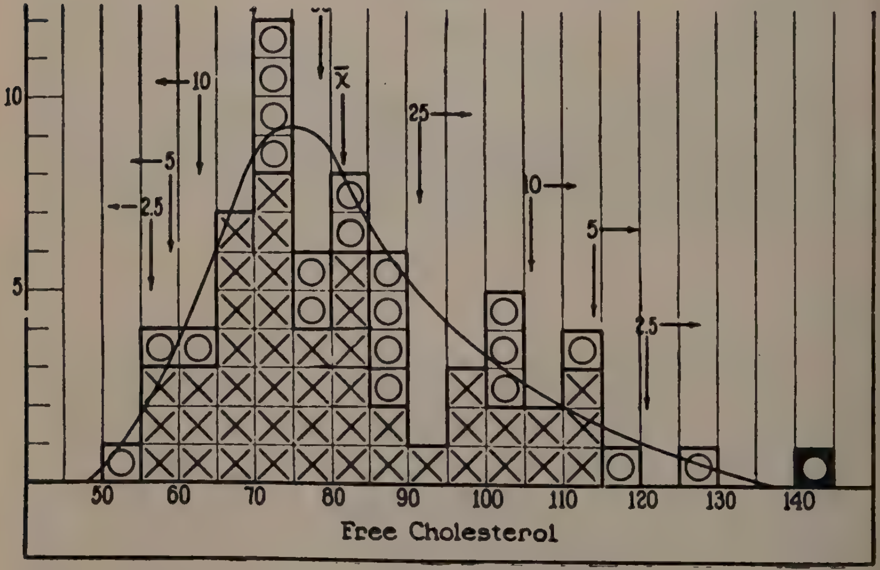

FIGURE 3

viduals were studied further and if the series of measurements were extended to include many diverse items, all of the 67 would be found to be "abnormal" in some respect. A substantial number of deviations are probably present in the makeup of each of us. Deviation is universal and, if an individual should turn up who is in the "normal range" with respect to every item in his makeup, he would be a most extraordinary and, in fact, a most "abnormal" individual.

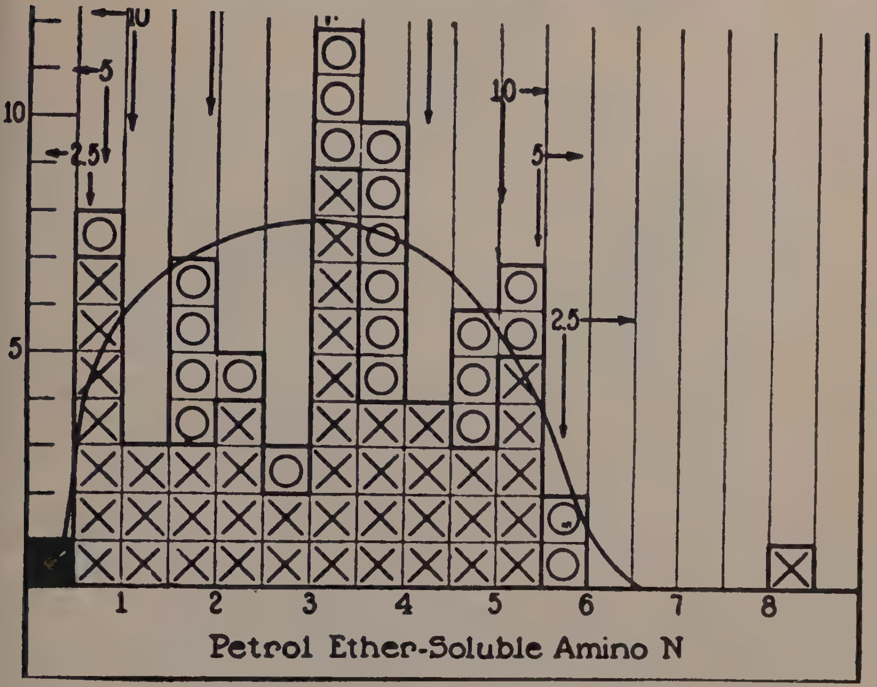

FIGURE 4

This point of view is further borne out by a consideration of the individual metabolic patterns[11] which we have studied at the University of Texas. Twelve individuals were measured several times with respect to 31 items: 5 taste thresholds; 12 salivary constituents, 14 urinary items. FIGURE 5 is a drawing, using polar coordinates, representing the hypothetical case of an individual who is exaclty average with respect to every one of the 31 items. In FIGURES 6–17 are the corresponding diagrams for each of the twelve individuals, using as the basis an average of the values obtained from each one.

In the pattern of *each* individual, there are from 2 to 5 items in which their distinctive values are one tenth or less that of the corresponding average values. In other words, the average, taken as a norm, is of the wrong order of magnitude for some items in the case of *every individual in the group*. In this study, every finding points to the nonexistence of the human being who is "normal" in all respects.

In considering the effects of environmental influences on human beings—food composition, temperature, light, infective agents, and drugs which may combat them, altitude, noise, housing and clothing, home and social influences, *etc.*—we are prone to think in terms of their effects upon the hypothetical "normal human being," and to disregard the biological variation which makes each of us a mosaic in which greater or lesser deviations are the rule rather than the exception.

In the field of nutrition, emphasis on the concept that people, as a rule, are

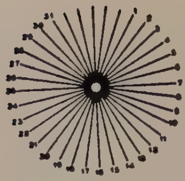

FIGURE 5

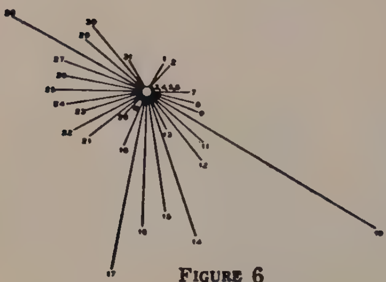

FIGURE 6

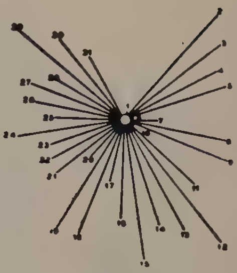

FIGURE 7

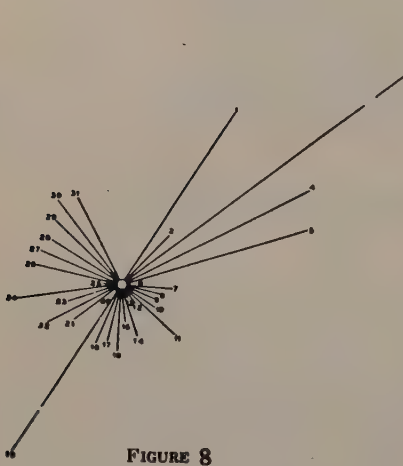

FIGURE 8

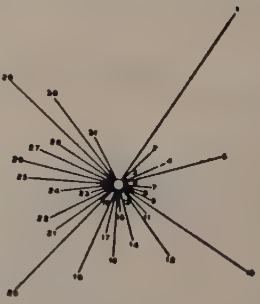

FIGURE 9

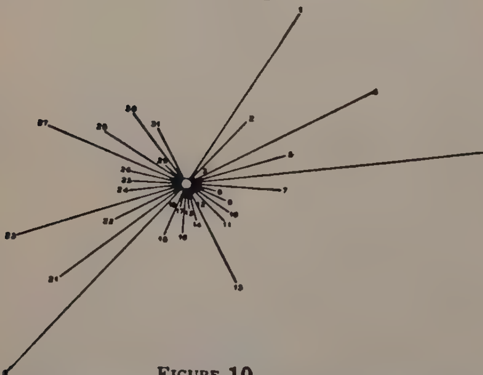

FIGURE 10

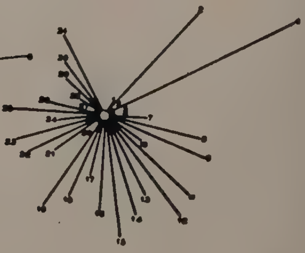

FIGURE 11

FIGURES 5–17. Taste sensitivity: 1. Creatinine; 2. Sucrose; 3. KCl; 4. NaCl; 5. HCl. Salivary constituents: 6. Uric acid; 7. Glucose; 8. Leucine; 9. Valine; 10. Citrulline; 11. Alanine; 12. Lysine; 13. Taurine; 14. Glycine; 15. Serine; 16. Glutamic acid; 17. Aspartic acid. Urinary constituents: 18. Citrate; 19. Base Rf.28; 20. Acid Rf.32; 21. Gonadotrophin; 22. pH; 23. Pigment/creatinine; 24. Chloride/creatinine; 25. Hippuric acid/creatinine; 26. Creatinine; 27. Taurine; 28. Glycine; 29. Serine; 30. Citrulline; 31. Alanine.

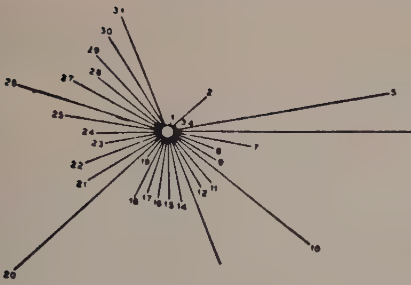

FIGURE 12

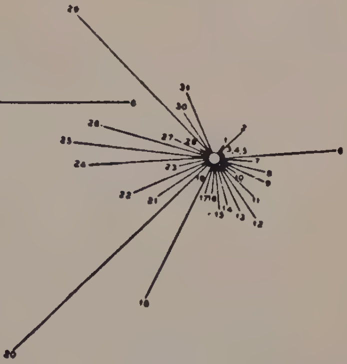

FIGURE 13

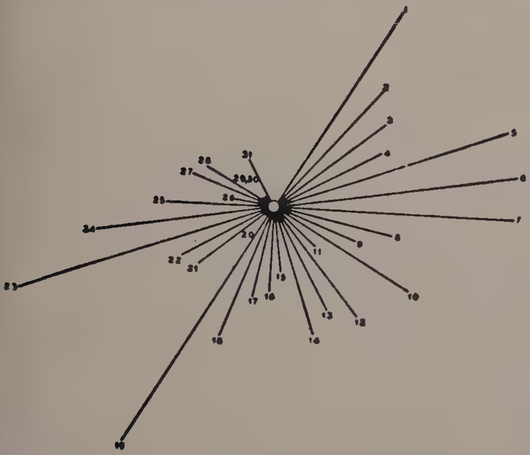

FIGURE 14

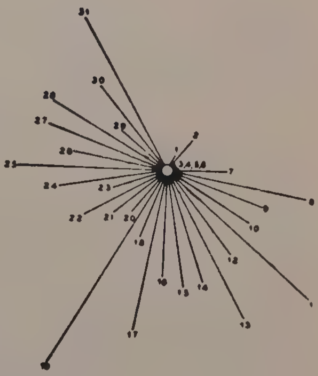

FIGURE 15

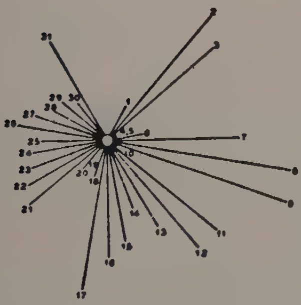

FIGURE 16

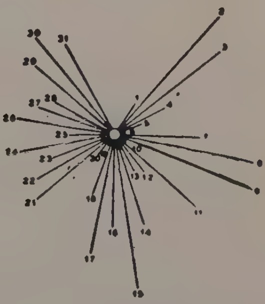

FIGURE 17

consistently normal is particularly pertinent. Indeed, if the population consists essentially of normal human beings who have substantially identical nutritional needs, then the genetotrophic concept about which I am to speak is invalid and must be completely disregarded.

In the light of modern biochemical genetics, however, this uniformity in nutritional needs cannot exist. Genes in a sense beget enzymes, and enzymes require substrates in the form of foods and the metabolites derived from them. Since the assortment of genes is distinctive for each of us, we must each have a distinctive pattern of enzymes and enzyme systems of varying efficiencies, and it follows, therefore, that our substrate needs are not the same, quantitatively speaking. The widespread prevalence of partial genetic blocks[4, 5] or their equivalent, makes it reasonable to suppose the nutritional needs of each of us for calcium, for leucine, for vitamin A, for riboflavin, *etc.*, are distinctive. The variabilities may be large or small, but they are inescapable. There is reason, I believe, to suspect that the deviations are much larger than has traditionally been thought. They certainly have not been studied sufficiently so they can be dismissed as inconsequential.

With this discussion as a background, it becomes easy to indicate the meaning of the genetotrophic concept. It is simply that some individuals, because of their genetic makeup, require more of certain nutrients than they are likely to get from ordinary food (especially the stored and processed food of an industrial civilization) and for this reason they tend to become deficient, and unwell as a result. If, however, such individuals are furnished an extra supply of the items which they individually need, their difficulties should disappear and their status should be as though the basic genetic peculiarities did not exist.

We have postulated that numerous diseases of which the etiology is obscure may have genetotrophic origins.[1, 2] Such diseases by definition are of genetic origin but are also nutritional (trophic) in nature and hence ideally may be eliminated by nutritional means.

A clear-cut case where the genetotrophic concept appears to apply is the disease alcoholism, which I wish to discuss briefly. Actually, the genetotrophic concept grew out of our experimental findings while we were studying the problem of alcoholism, and hence in this discussion I have, in a sense, placed the cart before the horse. This has been done deliberately, however, because the genetotrophic concept is much more far-reaching in its implications than anything that could be said about alcoholism, and I hope that the subject of alcoholism will not crowd the broader aspect of the genetotrophic concept out of your thinking. I do wish to make it clear, however, that our theorizing about the etiology of alcoholism grew very definitely out of experimental findings which we were at first at a loss to explain. We did not theorize first and then set out to find corroborative evidence.

We did, however, after a careful survey of the literature, come to the conclusion that potential alcoholics probably have inborn differences, which needed to be sought out.[12]

Our first attack consisted in a prolonged and detailed study[3] of the metabolic patterns of a group of alcoholics, who were not drinking at the time, and of a corresponding control group. The items investigated were more numerous,

TABLE 1
TABULATION OF HIGH AND LOW CHARACTERISTICS IN ALCOHOLICS IN ORDER OF DECREASING SIGNIFICANCE

High	Low
Sodium (S) (99)	
Hippuric acid (U) (99)	Gonadotrophin (U) (96)
Uric acid (U) (95)	Citrulline (U) (95)
Uric acid (S) (95)	
Thiamine (U) (94)	
Citrate (U) (92)	
Magnesium (B) (92)	
Pigment/creatinine (U) (91)	Phosphorus (B) (90)
Weak acids (U) (88)	Taurine (U) (88)
Sodium chloride (T) (88)	*Bacillus subtilis* (P) (88)

Numbers in parentheses are P values for difference between alcoholics and controls. (S = saliva, U = urine, B = blood serum, T = taste threshold, P = phagocytic index.

but similar to those depicted in FIGURES 6–17. Some blood analyses were included. The results of this study are summarized in TABLE 1. It will be noted that there are six items in which the alcoholics differ significantly at the 95 per cent level, or better, from the nonalcoholics. Alcoholics have, according to these findings, high sodium and uric acid content of the saliva, and high high hippuric acid and uric acid content of the urine, along with low urinary content of gondadotrophin and citrulline. The question of whether excessive alcohol consumption may have *caused* significant alterations in pattern is not a closed one, but we have several evidences bearing upon it. Identical twins (FIGURES 16 and 17) were found to have patterns very much alike,[11] indicating the importance of inheritance. Changes in diet do not change materially the patterns exhibited by individuals.[14, 15] Even small babies, living largely on milk, have distinctive patterns just as adults do.[16] Experimental animals exhibit highly distinctive excretion patterns when on identical diets,[7] and an extensive study of several closely inbred strains of rats shows clearly that these patterns have a genetic origin.[18] All these facts point to the conclusion that the alcoholics probably were deviates of the sorts indicated by their patterns *before* they began excessive alcoholic consumption.

The other method of attack on the problem of alcoholism was suggested by the work of Mardones and his co-workers,[19] who presented evidence regarding the existence of an antialcoholism vitamin which was called "Factor N." Our initial experiments[20, 21, 22] consisted of placing white rats from our colony (originating from a Wistar strain) in individual cages on a stock diet, and furnishing them with two drinking bottles, one containing water, the other 10 per cent alcohol. The positions of the two bottles were switched daily.

Experiments similar to this one had been done before by Richter[23, 24] and Mardones,[19] Brady and Westerfeld,[25] and others. The distinctive element in our study was that we ignored averages and concerned ourselves with the drinking behavior of *individual rats*. We found a high degree of individuality. Some rats drank considerable quantities of alcohol the first time it was offered and every day thereafter. At the other extreme, some rats were teetotalers. One rat in the original group drank just a little alcohol every day as long as the experiment lasted. Others drank a little at first and then after several weeks their consumption rose to moderate or high levels. Others increased their consumption to much higher levels in two or three weeks. In addition, some rats were spasmodic drinkers—they drank heavily for a day or two and then abstained for several days before indulging again.

Since these rats were on identical diets and their other environmental conditions were the same, the results suggested that heredity was an important factor in determining their individual drinking patterns. A study of other groups of rats, including six closely inbred strains (brother-sister mated for many generations), has given ample evidence to confirm this conclusion. Rats from different sources show wide differences in their drinking patterns and especially in their responses to nutritional supplements. Most inbred strains of animals, however, have not been bred for factors which cause uniformity in alcohol consumption and, hence, there is often wide intrastrain variation.

Further experiments confirmed the findings of Mardones and co-workers, as well as those of Brady and Westerfeld; namely, that dietary supplements decrease the alcohol consumption of rats. In fact, we found that the alcohol consumption of practically all the rats in our original colony could be brought to very low levels by administering the known vitamins, including vitamin B_{12}, and linseed oil as a source of unsturated fat acids, to animals on an otherwise standard diet. On a diet, marginal with respect to known B vitamins, substantially all the rats would drink alcohol at a high level; but when the deficient nutrients were supplied, alcohol consumption often dropped dramatically overnight and would continue at a low or even zero level thereafter.

When the experiments were shifted to the use of another colony of rats of Wistar origin, exactly similar results were obtained, except that a much larger percentage of rats were "refractory" and continued alcohol consumption even after all known nutrients were supplied in what was thought to be abundant amounts. There was not the slightest doubt, however, about the dramatic response observed in the case of many rats.

It is desirable to point out here that no two laboratories could hope to duplicate each other's detailed findings unless they took extraordinary precautions to use rats of exactly the same genetic origin. Very wide divergences could easily result from strain differences in the animals. It may be well to emphasize, however, that inasmuch as similar results have been obtained hundreds of times, there is not the slightest doubt but that dietary deficiencies in experimental rats bring about greatly increased alcohol consumption, and that meeting these deficiencies (which is not always easy or even possible in all cases) brings about a dramatic decrease in or even cessation of alcohol consumption.

Studies from our laboratory have sought to find out what particular vitamin deficiencies among the better known ones are most effective in inducing alcohol consumption in rats. It was found[26] that single deficiencies of thiamine, riboflavin, pantothenic acid, or pyridoxin are relatively effective in this respect, but that deficiencies of other nutrients, vitamin A, biotin, and choline are also somewhat effective, as judged both by the number of rats responding, and the magnitude of the response. Studies previous to this one had shown that vitamin B_{12} is certainly a factor and that unsaturated fat acids probably are. It is our conclusion that the intricate variations with respect to the quantitative aspects of the total metabolic stream, including all the eddies, are such that a considerable number of individual deficiencies may lead to the same crucial result, a failure to metabolize and get the energy out of the food.

One striking fact which these studies reveal is the existence of hitherto unknown nutrients which, in addition to all the others, are effective in preventing alcohol consumption or reducing consumption if it is already at a high level. For some animals these "unknowns" need not be given, presumably because they are not required at high levels.

It was on the basis of these original findings that the genetotrophic concept was evolved. It was presumed that each individual animal has its own metabolic peculiarities and, hence, from the quantitative standpoint, different nutritional needs. The existence of individual metabolic peculiarities in animals is amply demonstrated by their highly individual excretion patterns, when their food is the same.[17, 18] It was furthermore assumed that the rats which failed to consume alcohol when fed a stock diet were adequately nourished by the stock diet. Those individual animals which drank alcohol heavily while on a stock diet were initially somewhat deficient due to the possession of unusually high requirements for items not furnished in sufficient quantity by the stock diet. When we shifted to a different group of animals, many of which were refractory to the treatment which was effective for the original rats, we regarded this as a manifestation, on the part of the "refractory" rats, of a relatively high requirement for something of an unknown nature, which we were unable to supply in a routine manner in sufficient amounts.

Why vitamin or other nutrient deficiencies should induce an increased appetite for alcohol is not clear, but it seems reasonable to assume that it is connected with the fact that alcohol is a ready source of energy and, in spite of its unfortunate side effects, can be utilized without as much involvement of intricate metabolic machinery as other forms of fuel. In keeping with this idea is the fact that, when similar choice experiments are set up involving other combustible "fuels," methanol, propanol, butanol, or glycerol in place of ethyl alcohol in one of the drinking bottles, the animals fail to consume an appreciable amount of any of the substitutes.[27] In comparable experiments using glucose solutions, animals exhibit a high degree of individuality in the amounts they consume, but our experiments have not been extensive in this connection.

The recent studies of Greenberg and Lester[28] show that giving rats access to sugar solutions causes a decrease in alcohol consumption. This finding complicates the picture, but does not invalidate, we believe, the idea that alcohol consumption is determined by genetics and by nutrition. It does make it appear that the correspondence between alcohol consumption in rats and alcoholism in human beings is not as close as has sometimes been assumed. This may be due to differences in the metabolism of the two species. It is well known, for example, that rats have synthetic powers (*e.g.*, for producing ascorbic acid) which humans do not possess.

The fact that nutritional factors are of paramount importance in determining the alcohol consumption of experimental rats does not rule out the effect of psychological stresses. We have carried out a limited number of experiments[29] which indicate that subjecting animals to flashing lights and continuous noise can increase alcohol consumption. The most interesting experiments dealt with "teetotalers," rats which on stock diets failed to drink alcohol. Some of these (but not all) could be induced to drink appreciably when subjected to the stresses; but generally it was only temporary, and the drinking declined when the animals became accustomed to the "annoyances." It is well known that psychological stresses bring about physiological changes, and it seems reasonable to suppose that the intricate stream of metabolism could be so changed by psychological stress as to augment the needs for specific nutrients. Thus, even when psychological factors are involved, the basic change may be metabolic and therefore subject to nutritional influence.

Obviously, the next step is the human application of these findings. Is it possible, by the use of nutritional supplements, to abolish or reduce to manageable levels, the craving for alcohol exhibited by certain human beings? The answer is, I believe, a certain and unequivocal affirmative.

It is needless to say that the gamut of problems which arise in connection with consumption of alcohol—economic, social, health—are not all to be solved by any single stroke. The one problem with which we have been primarily concerned is that of the drinker who cannot control his drinking, the man or woman who is seemingly forced by the first drink to take another and another until he or she becomes incapacitated for useful work. It is our opinion, and the findings support it, that such craving is essentially physiological and very often beyond intellectual control.

I have come in personal contact with several individuals, all of whom had a terrific craving for alcohol (in some cases of many years' duration) who have been transformed by the use of dietary supplements into individuals who could drink moderately without excess, not for a period of days or weeks, but actually for a year or more.[30] The first individual was treated about three years ago and, as long as he has continued the recommended dietary supplements, he has been able to avoid drunkenness and, at the same time, regularly to drink beer, his favorite beverage.

Treatment of alcoholism is complicated by the fact that, for some individuals, the enjoyment derived from drinking is so great that they would as soon part with their right arm as with this pleasure. This is by no means true of all alcoholics. Some would gladly give up alcohol entirely if the physical craving allowed them to do so. People, in general, have to gain some pleasure from life, otherwise they will not want to live, and the alcoholic who looks to alcohol as his supreme pleasure in life and, at the same time, has an intense physical craving for liquor, has a double problem, and nutritional supplements can only abolish the craving. They cannot furnish him new motives for living. In a book now in press,[31] I have presented evidence which shows that even people with similar training and similar educational leanings have extremely diverse "want patterns" and derive their satisfactions from life in entirely different ways. The alcoholic is fortunate if he can cultivate reasons for living which do not include alcohol. For such individuals, I believe there is real hope for the future. If nutritional supplements now available will not curb or obliterate the craving, we may hope that future progress will make possible its abolishment.

To one not experienced or even initiated in the field, it might seem a simple matter to test a nutritional supplement for its efficacy in the treatment of alcoholic craving. Actually, however, organized provision for testing *bona fide* scientific proposals in the field of therapeutics is nonexistent and, more often than not, such proposals must be considered and passed upon in a haphazard fashion by practicing physicians who have little or no time or inclination toward research.

In connection with our alcoholism treatment, consisting of a carefully balanced assortment of nutritional supplements, there has been one group which is making a systematic controlled test, using placebos to eliminate psychological effects. They have made a preliminary report[32] which corroborates the results of our uncontrolled tests. Unfortunately, from the standpoint of many alcoholics who may not be induced to give the supplements a trial, the results with human beings are too much like those with rats. The particular assortment suggested *will not eliminate the craving in all individuals.* It is highly successful with some, only moderately successful with some, and not at all successful, so far as we know, with others. What the percentages are no one knows. The first experiments indicated about 50 per cent success.

Here we come to another difficulty. Unless physicians have some faith in the nutritional supplements and transmit this faith to their patients, the supplements will not be taken regularly, and of course they can do no good unless they are taken. Physicians have learned by experience that unless patients have a strong urge to do so, they will not take anything that requires attention three times a day. So, in any trial run, the number of patients *who are supposed to have taken medication* will be materially greater than the number *who actually take it.*

Before an adequate country-wide trial of nutritional supplements as a means of abolishing alcohol craving can be made, it is essential that more physicians understand the inescapable scientific background of the genetotrophic concept, and become receptive to the idea that, in alcoholism as well as in many other diseases, genetic deviations from norms are crucial. We can never understand diseases in which this is true as long as we center our attention on "man," who is supposedly thoroughly normal. The "normal" man does not exist.

I do not want what I have said about alcoholism, a subject which elicits a tremendous variation in opinion, to detract from the central theme of my discussion; namely, the broad basis and significance of the genetotrophic concept.

If this concept is valid, we are not left helpless with our genetic peculiarities, some of which may cause severe trouble. We can overcome many by nutritional needs, provided our knowledge is adequate. Insight into how to overcome these difficulties demands, however, that we pay careful attention to deviations instead of smoothing them away by statistical methods.

One of the concepts which physicians, nutritionists, and others often entertain is that of "the normal well individual." We have already given evidence which denies the existence of individuals who are normal in a thoroughgoing fashion. I believe the case is also strong against the existence of individuals who are thoroughly well. Certainly, the great majority of people that I know are unwell in one way or another, whether they consult physicians regularly or not. Occasionally, one hears an adult or even an elderly person say, "I have never been sick a day in my life." Certainly, if their boastings are true, such individuals are rare enough to be outside the range of "normality," so far as the general population is concerned.

In a great many cases, it seems reasonable to suppose, the widespread manifestations of unwellness, lack of vigor, lack of adjustment, failure to mature, *etc.*, are based upon the fact that each of us is a deviate in a number of different ways. Some of the deviations which we possess may cause trouble and must be understood before they can be managed. Many of the deviations, however, are an inexorable part of life and cannot and should not be dispensed with. Even these desirable deviations, however, need to be understood if we are to understand ourselves.

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Comments on the Paper

Doctor Daniel Feldman, *New York*: The evaluation of any treatment regime directed against the disease, chronic alcoholism, is fraught with many pitfalls. The peculiar nature of the illness manifests itself in great variations in drinking patterns, and the personality of the chronic alcoholic is such that he may respond with a period of abstinence to almost any procedure used. Unfortunately, these periods invariably end with a resumption of the drinking. I think that, in order to evaluate the effectiveness of any therapy, the patient's previous drinking pattern must be clearly known, and a sufficiently long time must elapse before any conclusions can be drawn. I should think that a minimum period of observation of at least a year is necessary before an evaluation of the success of a therapy can be made.

The very important question of inadvertent psychotherapy arises in any critical evaluation of a therapeutic regimen. The part played by such relationships as suggestibility, support, and the "magic" value of the material used, must be honestly assessed. One cannot ignore the fact that, because of his personality makeup, the alcoholic is positively looking for such psychological aids. As a matter of fact, it is extremely difficult, if not virtually impossible, for the person dispensing the medication to divorce himself from a psychological relationship of some sort. Obviously, this makes it impossible to eliminate this factor in the evaluation of any treatment regime aimed at curing or arresting the disease.

Despite this, however, I think that a therapy such as Dr. Williams' nutritional one can be honestly evaluated if certain things are borne in mind; for example, the use of the patient's past alcohol pattern as a control. If a significant difference is observed between his drinking pattern before and after the nutritional regime, and if an equal amount of psychological help was given in the past; then, one might suspect that the improvement noted might in some way be related to the use of the nutritional substances. I am aware that this statement could be criticized and that it could be pointed out that the individual managing the nutritional therapy might, because of his personality, supply certain psychological aids that the alcoholic patient did not have before. A controlled experiment making use of identical placebos and carried on for a sufficiently long period of time would be of major importance in determining the part played by nutritional treatment. Because of the great number of variations in drinking patterns, and because of the peculiar psychological makeup of the alcoholic, one must be extremely wary in interpreting the results of any particular form of therapy.

As regards Dr. Williams' report on a patient who became a social drinker following nutritional therapy, one can say that it certainly is an unusual result, and it would be very important to follow this individual for a long period of time. Not infrequently, one sees alcoholics who can "drink socially" for periods of time, but invariably revert to pathological drinking.

Another point which is of great interest is Dr. Williams' deduction that abnormal alcohol intake is associated with genetic nutritional abnormalities and, consequently, with nutritional deficiencies. Why should alcohol, a substance which does not, as far as we know, correct any of the nutritional deficiencies which may be present, be selected by such individuals? The psychological effects of alcohol, on the other hand, are well known and, if one presumes that the alcoholic chooses this substance for its inhibiting effect on cerebral processes, one has at least a logical reason for its use. The emotional disturbances and psychological difficulties of alcoholics are well known, and most carefully studied data point to the fact that these difficulties have pre-existed the alcoholism itself. I should like to know Dr. Williams' feelings concerning these psychological difficulties and their relationships to the genetic–nutritional abnormalities which he postulates.

I should like to summarize briefly our experience with Dr. Williams's method of treatment.

A group of 26 patients was treated at the outpatient clinic with nutritional supplements, weekly interviews and, in a good number of instances, was attending A. A. as well. The study was begun Oct. 20, 1952, but many of the group were not seen until several months later, being added as they appeared at the clinic. Fifteen patients in this group are still being seen with regularity and are not drinking, as far as we know. Ten of the group have failed to return, and three are known to be drinking heavily. Follow-up of the remaining seven has, as yet, been unsuccessful. One patient is not attending the clinic because of job interference, but reports by telephone. When last heard from, he was having no drinking problem and was continuing nutritional therapy. Although there was loss of control, this patient would not be considered even a moderately severe alcoholic.

All of the 26 patients studied reported a sense of well-being while on the nutritional regimen.

A great deal of psychotherapeutic relationship was present in this group, and our experience shows that it is practically impossible to avoid this. If one cuts the patient off short, a strong resentment and hurt to the imagined rebuff results, and the patient almost invariably drops out. However, inasmuch as most of these patients had failed to control their drinking under similar supportive therapies in the past, we were curious to see whether the inclusion of regimen to correct any nutritional deficiencies would make supportive therapy any more successful.

Most of the ten failures occurred in patients who were under treatment longest, and it may be that many of the 15 patients still in treatment have not been observed long enough for even the most preliminary of evaluation. It may be significant that the ten failures occurred in so short a period as four months.

Three patients were treated intensively at another clinic. Treatment began in October, 1952, with a potent nutritional supplement and visits were held down to the minimum necessary for accurate observation. Such visits took place at three or four week intervals. The supportive value of such visits became apparent from the patients' own remarks.

One of the patients was a steady pathological drinker, whose inebriety was interrupted by short periods of partial sobriety. He had followed this pattern for many years. He accepted the nutritional regimen eagerly and was high in praise of what it had done for him. His drinking habits showed no change whatsoever, and eventually he did not return to the clinic. It is interesting to note that this patient stated quite frankly that the interviews were very helpful, and he would call for additional appointments between his regularly scheduled visits.

The second patient was a severe periodic drinker, going on "benders" once or twice a year. He abstained between bouts. He reported an increased sense of well-being during the nutritional regimen. Four months after beginning treatment, he went on a severe prolonged drinking bout, exactly the same as those prior to therapy. For two weeks prior to this, he reported that he could drink an occasional beer.

The third patient is a compulsive periodic drinker with no control. His only sobriety was attained through taking church pledges and, usually, he anxiously awaited the end of such a period and promptly went on a bender. The onset of nutritional therapy coincided with such a pledge. He claims he generally feels better and, although he thinks about drinking, there is no compulsion. The pledge has not been in enforcement for two months now, and the patient is still not drinking, the first time in his experience that this has happened. Although he feels the "pills" have helped, he states that the fact that he has had a physician at the clinic to whom to come has been a great help and relief to him, and he looks forward to his visits. He has never before had any treatment.

NUTRITIONAL ASPECTS OF CIRRHOSIS IN ALCOHOLISM—EFFECT OF A PURIFIED DIET SUPPLEMENTED WITH CHOLINE*

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The association of alcoholism and cirrhosis of the liver was observed well over a century ago and is now generally accepted. That alcohol played an etiological role through a direct toxic action on the liver was the natural assumption to be derived from this observation until the demonstration that fatty cirrhosis similar to that seen in man could be produced in animals by means of low-protein diets.[1, 2] Moreover, attempts to produce cirrhosis in animals by the administration of alcohol supplements to an adequate diet have been for the most part unsuccessful.[3] As a result, the concept of cirrhosis in the alcoholic has been reoriented toward the nutritional aspects; so much so, in fact, that many consider this disease simply as a nutritional deficiency.

The evidence that cirrhosis in the alcoholic is a manifestation of a nutritional deficiency is not conclusive. To be sure, the reports from various parts of the world which implicate malnutrition unassociated with alcoholism are impressive.[4, 5] But most of the patients with fatty cirrhosis in this country are chronic alcoholics; and even though dietary intake is usually poor during periods of imbibition, alcohol has not been exonerated as an important causative factor, although there is little positive evidence to incriminate it. Another argument for a nutritional basis for the cirrhosis of the alcoholic stems from the therapeutic efficacy of the regimen proposed by Patek and Post,[6, 7] which emphasizes a nutritious diet. But the success of this regimen in alcoholics drinking up till the time of admission to the hospital in itself cannot be attributed to the resumption of an adequate diet, as withdrawal of alcohol and other influences incident to hospitalization are incurred simultaneously.

Probably the most convincing evidence against an hepatotoxic action of alcohol has been the demonstration that the administration of alcohol in addition to a nutritious diet to chronic alcoholics with liver disease did not prevent hepatic functional and histological improvement in one patient[8] or exacerbate the liver disease of four patients already treated even though given for as long as 6 to 18 months.[7] Whether the alcohol consumed by these patients was quantitatively and qualitatively the same as that ingested before hospitalization would not be ascertainable.

Thus, although liver disease is certainly related to alcoholism, it is still not clear whether the liver disease of the alcoholic is a result of alcohol poisoning, nutritional deficiency, or both.

The discrepancy in our knowledge of the nutritional aspects of cirrhosis in the laboratory animal as compared to man is comprehensible and is attributable

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* This work was done under the sponsorship of the Commission on Liver Disease, Armed Forces Epidemiological Board. It was supported, in part, by the Office of the Surgeon General, Department of the Army; and, in part, by a grant from Merck and Company, Inc., Rahway, N. J., to Harvard University.

† On assignment from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

largely to the greater limitations of clinical investigation. As a result, we have found it necessary to approach the problem of cirrhosis in the alcoholic in a negative manner. This entails a study of the effects of nutrients on the course of the liver disease and, admittedly, is limited in establishing cause.

In order to determine the effect of a dietary regimen on the course of the liver disease, it is almost mandatory to study a patient with active disease, so that definite changes in the hepatic disturbance, in one direction or the other, can be discerned over a relatively short period of time. We have found the most useful indices of activity in the cirrhosis of the alcoholic to be the serum bilirubin and urine urobilinogen concentrations and prefer some elevation of both in the patients to be studied. The criteria that we have generally used to evaluate the course of the liver disease fall into two main categories: (1) "hepatic function" tests; and (2) hepatic histology. Measurement of hepatic size is so inaccurate that the small changes expected in short-term studies might easily be missed.

One of the principal difficulties in the evaluation of diets and dietary supplements in chronic alcoholics with active liver disease is the rapidity with which these patients usually improve, in our experience, following hospitalization and consumption of even meager diets. For this reason, many studies dealing with the comparative therapeutic efficacy of diets and dietary supplements in such patients have been relatively unrewarding. Moreover, even if a supplementary nutrient does not appear to augment the therapeutic value of a given diet, it is nevertheless quite possible that a deficiency of that nutrient was responsible for the liver disease. Rather than compare the therapeutic value of various diets and dietary supplements, therefore, it is first imperative to dissociate the effects of diet from the effects of alcohol withdrawal and hospitalization on the course of the liver disease, *i.e.*, to determine whether alcohol withdrawal and hospitalization in themselves might ameliorate the liver disease. In this respect, the rapid improvement referred to above is advantageous, as it permits the short-term studies necessitated by the intolerability of markedly deficient diets. If it could be shown that alcoholics with active liver disease did not improve on a basic diet despite alcohol withdrawal and hospitalization, but did improve on a more adequate diet, then it could be concluded that one or more constituents of the more adequate diet was responsible for the improvement. Individual dietary factors could then be added to this basic deficient diet to evaluate the effect on the liver disease. Any factor present in food which proves efficacious might seriously be considered to play an important etiological role in the genesis of the liver disease.

This method of approach using deficient diets has been employed in this laboratory for several years. The results of these investigations are reported herewith; those published being summarized, those unrecorded presented in more detail.

In 1949, Eckhardt, Faloon, and Davidson[9] studied the effects of a protein-free diet supplemented with intravenous amino acids on the course of four chronic alcoholics with active cirrhosis. Although the basic diet was essentially devoid of protein (0.1 gm. of nitrogen daily), it was shown to be capable of maintaining nitrogen balance and body weight when 85 gm. of purified casein were added daily as the sole source of protein.[10] It contained no source of the vitamin B complex except choline (30 to 100 mg. daily). The four patients were provided with the basic diet plus a multiple vitamin capsule (containing vitamins A, C, D, B_1, B_2, niacin, B_6, and pantothenic acid) and a 500 to 1000 mililiter infusion of a ten per cent solution of amino acids daily. The average daily caloric intake ranged from 1700 to 4500 calories. While on this regimen for a period of from two to four weeks, all of the patients exhibited a progressive decline in serum bilirubin; two showed a decrease in the size of the liver and spleen; and in the one patient who was biopsied serially, there was a decided improvement in hepatic histology. In one of the patients, moreover, the liver disease improved despite negative nitrogen balance.

In a subsequent study, Eckhardt *et al.*[11] followed three similar patients for from 8 to 13 days on the same basic diet with the same added vitamins, but without the supplementary amino acids. Each of the patients showed an improvement in "liver function" tests and a decrease in liver size. Liver biopsies performed at the start and finish of the protein-free diet period revealed no apparent change in fat content (by histology), except in one patient who received added choline for six days (12 gm. of choline chloride daily). These results clearly demonstrate that rapid improvement in the liver disease can occur in alcoholics while they are consuming a diet essentially devoid of protein. The possibility arises that this improvement was related either to the alcohol withdrawal and hospitalization, to the protein-free diet, or to both. It is noteworthy, however, that the three patients described had stopped alcohol intake and increased food consumption for from 4 to 7 days before the study began. Consequently, another explanation for the improvement must be considered, *i.e.*, that these patients may have received enough of one or more beneficial dietary factors during the period just prior to study to initiate and even perpetuate recovery.

To obviate this difficulty, Phillips, Gabuzda, and Davidson[12] studied three patients with active cirrhosis who were allegedly drinking up till the time of study, which started at the time of admission to the hospital. Evidence for this in two of the patients was the presence of Wernicke's syndrome. The patients were given no sustenance after admission except for a purified diet, consisting solely of solutions of glucose and minerals, which was continued for from 8 to 10 days. One patient received thiamine, and another thiamine plus multiple vitamins in addition to the diet. In contradistinction to the findings of Eckhardt *et al.*,[11] none of these three patients showed significant improvement in hepatic function, size, or fat content (by histology). After institution of an adequate diet for a smilar period, there was improvement in hepatic function in all three patients and a decrease in hepatic fat in two, the third patient not showing a significant diminution until the biopsy taken after two weeks of the diet. The failure to improve on a purified diet with subsequent improvement on an adequate diet for a similar period implied that there was something in the adequate diet responsible for the amelioration of the liver disease. These results would tend to relegate alcohol withdrawal and hospitalization to subordinate influences in this improvement. The failure to improve on this purified diet, moreover, affords the opportunity to assess the efficacy of individual nutrients added to this diet on the course of the liver disease. On the basis of the animal studies, choline and methionine would be logical factors to try. Studies of this sort using choline have been carried out and the results follow.

Five chronic alcoholics with evidence of active liver disease, who were drinking heavily and eating poorly until the time of admission to the hospital, were followed on the Thorndike Metabolic Ward. No complicating illnesses were encountered except in one patient (H. C.), who developed transient fever associated with a moderate degree of delirium tremens. After admission measurements, the patients received no sustenance other than the purified diet of glucose and saline in four cases and glucose solution only in the one patient (C. W.) with evidence of fluid retention. While on the purified diet, which was continued for from 4 to 10 days, each patient consumed approximately 400 gm. of glucose (1600 calories), 4 gm. of sodium chloride (except C. W.), and 2000 to 2500 mililiters of fluid daily. All of the patients found this diet highly unpleasant but tolerated it. Three patients had diarrhea (J. M., J. D., C. W.) which stopped within a few days after discontinuing the purified diet. In addition to the purified diet, each patient received choline* orally daily. This supplement was discontinued at the cessation of the purified diet period except in one patient (H. C.), in whom it was continued for 20 days thereafter. One patient (J. D.) received thiamine while on the purified diet because of the diagnosis of Wernicke's syndrome.

Following the purified diet period, a diet containing approximately 1600 calories, 50 gm. of protein, and about the same amount of salt and water daily as was in the purified diet, was instituted in five patients. In three of these, the caloric and protein contents of the diet were raised to about 3000 calories and 75 gm., respectively, for a subsequent period.

The measurements of hepatic function and size made on admission were repeated at appropriate intervals. The methods for determing bromsulfalein retention, serum bilirubin, thymol turbidity and flocculation, and urine urobilinogen and bile are given in a previous report.[12] Serum alkaline phosphatase† was measured by the method of Bodansky,[13] serum total cholesterol‡ as described by Schoenheimer and Sperry[14] and by Abell *et al.*,[15] and serum phospholipid‡ according to Schmidt *et al.*[16] Liver size was estimated by measuring the distance of the liver edge below the right costal margin in the midclavicular line on maximal inspiration. Liver biopsies were performed on admission on the five patients and repeated serially on four. All biopsies were done transthoracically using the Vim-Silverman needle and were generally uneventful. For serial biopsies, an attempt was made to insert the needle at the site of the previous puncture.

The results on each patient will be presented separately.

Case Studies

Case 1. Patient J. M. was a 51-year-old white male chronic alcoholic of 30 years duration who was hospitalized 10 to 12 years prior to admission for jaundice, anorexia, and weakness, and was told at that time that he had a "very slight cirrhosis." Two years before admission, his alcohol consumption was augmented and food intake concomitantly decreased. For the three weeks before entry, he felt weak and anoretic, ate very little, and decreased his alcohol intake somewhat. On admission, his liver edge was 14 cm. below the right costal margin, sharp but not very firm, and only slightly tender. There was no definite edema, ascites, icterus, splenomegaly, or spider telangiectasia.

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* Choline dihydrogen citrate (Abbott Laboratories, North Chicago, Illinois).

† The authors are grateful to Doctor Stephen Maddock, Surgical Research Laboratory, Boston City Hospital, in whose laboratory the determinations were carried out.

‡ The authors are grateful to Doctor David Hurwitz, Chief of Diabetic Clinic, Boston City Hospital, in whose laboratory the determinations were carried out.

Results (TABLE 1, FIGURES 1 and 2): During the first ten days of hospitalization, the patient received the purified diet and 6.5 gm. of choline dihydrogen citrate orally daily. Over this period, the bromsulfalein retention, serum bilirubin, and urine urobilinogen, which were all abnormal on admission, decreased progressively. The thymol turbidity, which was very high on admission, likewise declined considerably, but since the thymol flocculation was consistently negative, this change may have been related to the steady decrease in serum phospholipid and total cholesterol, which were also quite high on entry. The liver edge was thought to recede 2 cm. during this time. Following the ten days of the purified diet, the patient received for the next 30 days the 1600 calorie-50 gm. protein diet but no added choline. The improvement in the above measurements continued so that after 30 days of this diet, those remaining abnormal were only a bromsulfalein retention of 7 per cent and a hepatomegaly to 8 cm.

The admission liver biopsy showed a large amount of fat with minimal fibrosis. There were occasional polymorphonuclear leucocytes but no intracellular hyalin was seen. Many of the cells appeared frayed and the nuclei were small and dark. Repeat biopsies done after both 7 and 10 days, respectively, of the purified diet and choline disclosed a definite but not marked decrease in fat and many cells with two nuclei, suggesting regeneration. The cells were less frayed and the nuclei were more normal in appearance. A biopsy take after 10 days of the 1600 calorie-50 gm. protein diet revealed still less fat, and one taken after 30 days of this diet showed only a slight to moderate amount of fat, many binucleated cells, and significantly more fibrous tissue than was apparent on the previous biopsies.

Case 2. Patient S. C. was a 48-year-old white male chronic alcoholic of 26 years duration who usually drank intermittently in boutss lating 2 to 3 months and separated by intervals of abstinence lasting 4 to 9 months. He had been drinking steadily and eating little for 8 months prior to admission. During the two weeks before admission, he became quite anoretic and consumed very little food but continued drinking. He claimed that he took 1 to 2 multiple vitamin pills* daily for 1 to 2 months prior to admission. No history of previous hepatic disturbance could be elicited. The admission physical examination revealed facial telangiectases, palmar erythema, and an enlarged liver with a moderately firm tender edge, felt about 15 cm. below the right costal margin. No definite edema, ascites, icterus, splenomegaly, or spider telangiectasia was made out.

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* "Unicaps" (Upjohn). Each capsule contains Vitamin A, 5000 U; Vitamin D, 500 U; ascorbic acid, 37.5 mgm.; thiamine hydrochloride, 2.5 mgm.; riboflavin, 2.5 mgm.; pyridoxine hydrochloride, 0.5 mgm.; calcium pantothenate, 5 mgm.; nicotinamide, 20 mgm.; folic acid, 0.25 mgm.; and Vitamin B_{12} activity, 1 μgm.

TABLE 1

CLINICAL AND LABORATORY DATA OF FIVE CHRONIC ALCOHOLICS WITH CIRRHOSIS OF THE LIVER WHO RECEIVED A PURIFIED DIET AND CHOLINE FOLLOWED BY A MORE ADEQUATE DIET FOR THE SAME PERIOD

All measurements were made at the end of the specified days of diet except the "Initial" measurements which were made on admission to the hospital.

Patient	J. M.			S. C.			J. D.			C. W.			H. C.		
	Initial	Purified diet and choline‡	1600 cal.-50 gm. prot. diet	Initial	Purified diet and choline‡	1600 cal.-50 gm. prot. diet	Initial	Purified diet and choline‡	1600 cal.-50 gm. prot. diet	Initial	Purified diet and choline‡	1600 cal.-50 gm. prot. diet	Initial	Purified diet and choline‡	1600 cal.-50 gm. prot. diet and choline‡
Days of diet		10	10		9	9		7	7		5	5		4	4
Bromsulfalein % retention	40	21	13	43	34	22	61	60	53		38	39	33	34	35
Serum bilirubin, mgm./100 cc. "1 min."	1.5	0.40	0.25	1.3	0.67	0.25	5.2	6.1	3.4	1.7	2.5	1.7	4.5	5.8	6.7
Serum bilirubin, mgm./100 cc. Total	3.2	1.6	0.74	2.5	1.4	0.74	10.8	11.3	6.8	3.7	5.1	3.7	9.6	12.0	13.8
Thymol turbidity*	>8.7	3.1	2.9	3.3	3.0	2.6	8.0	6.9	4.4	2.9	2.9	2.3	>8.7	>8.7	8.5
Thymol flocculation (0–4+)	0	0	0	1+	0	0	2+	2+	2+	0	0	0	3+	3+	3+
Urine urobilinogen	1:512	1:128	1:32	1:512	1:16	1:32	1:256	1:4	1:4	1:128	1:128	1:128	1:128	1:512	1:128
Urine bile (0–4+)	2+	0	0	1+	0	0	2+	3+	2+	3+	3+	±	4+	4+	4+
Serum alkaline phosphatase††	—	—	—	7.3	5.1	5.8	11.0	8.3	5.3	2.9	3.0	3.3	10.8	6.2	10.4
Serum cholesterol, mg./100 cc.	1041	519	363	184	182	—	378	327	327	83	74	75	615	513	501
Serum phospholipid, mg./100 cc.	835	378	265	141	149	—	532	336	330	32	35	31	508	531	560
Liver size**, cm	14	12	11	—	—	—	7	8	8	5	7	6	11	11	11

‡ See text for quantities.
* Maclagan units.
†† Bodansky units.
** Below costal margin in midclavicular line on maximal inspiration.

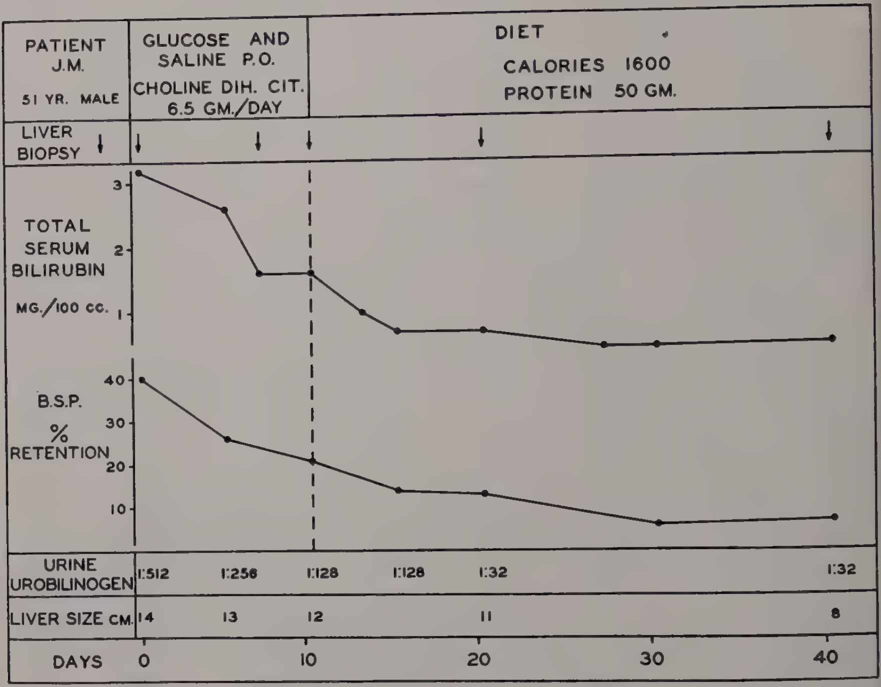

FIGURE 1.

Results (TABLE 1, FIGURES 3 and 4): The patient was maintained on the purified diet for 9 days and, in addition, received 3.25 gm. of choline dihydrogen citrate daily for the first 5 days and 6.5 gm. daily for the next 4 days. During the first 5 days, there was a drop in urine urobilinogen, but no significant improvement in the bromsulfalein retention, serum bilirubin, or thymol turbidity and flocculation. During the next four days, however, all of these tests changed toward normal. He was then given a 1600 calorie-50 gm. protein diet for 20 days followed by a 3000 calorie-75 gm. protein diet for 19 days. Improvement in the tests continued so that at the end of the study, bromsulfalein retention was 10 per cent while the other tests were normal. The patient's obesity made it difficult to palpate his liver edge with any certainty and thus prevented reliable estimation of liver size.

The initial liver biopsy revealed a great deal of fat and fibrosis with abundant bile duct proliferation. In addition, there was a moderate polymorphonuclear leucocyte infiltration and a small amount of intracellular hyalin. Many cells appeared frazzled with indistinct outlines and the nuclei were generally pyknotic. There were occasional areas with binucleated cells. After 9 days of the purified diet and choline, the hepatic fat appeared to be decreased; the number of polymorphonuclear leucocytes seemed to be fewer, but the intracellular hyalin, although involving few cells, was more prominent than on the previous specimen. The cellular architecture was considerably improved and

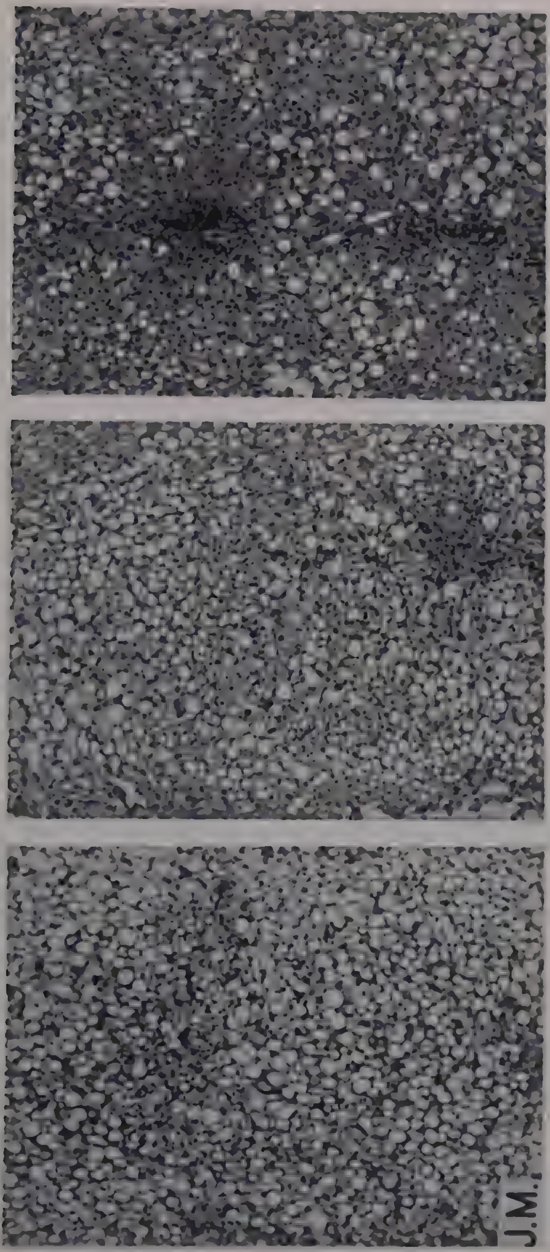

Figure 2. Biopsies for patient J. M. The biopsies are in chronological sequence from left to right. The first biopsy was performed on admission; the second, after ten days of the purified diet and choline; and the third, after ten subsequent days of the 1600 calorie 50 gram protein diet. Hematoxylin and eosin stain. × 50.

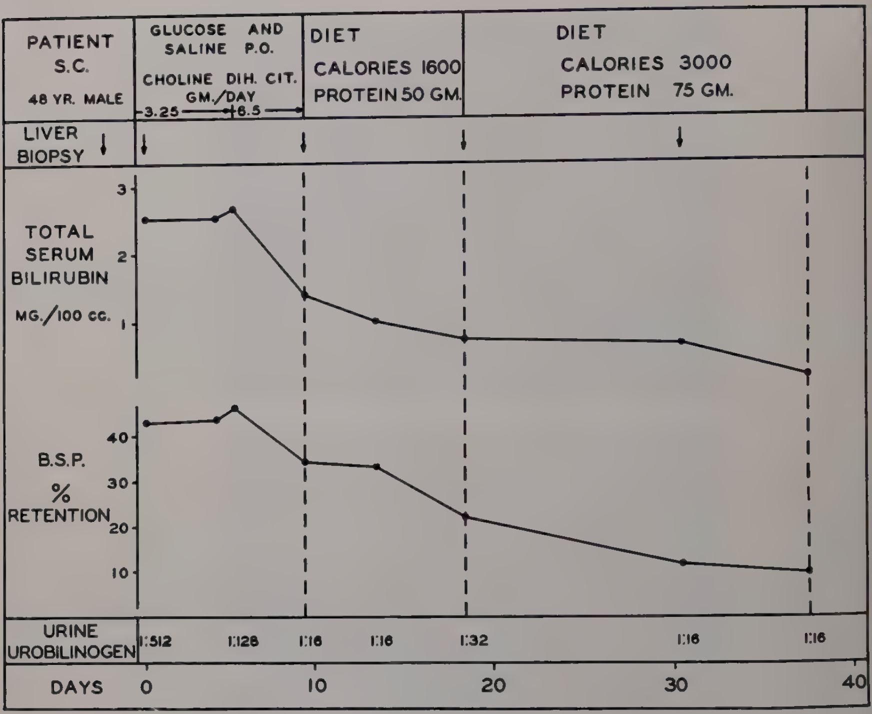

FIGURE 3.

many binucleated cells were evident. A repeat biopsy was performed following 9 subsequent days of the 1600 calorie-50 gm. protein diet and showed considerably less fat, essentially no polymorphonuclear leucocytes, rare intracellular hyalin and many binucleated cells. The final biopsy specimen was obtained after 12 days of the 3000 calorie-75 gm. protein diet and disclosed a minimal quantity of fat, no polymorphonuclear leucocytes or hyalin, but a severe degree of fibrosis, more marked than on the previous specimens.

Case 3. Patient J. D. was a 54-year-old white male who drank steadily for about 36 years except for a period of abstinence of 7 to 8 years, starting 20 years before. He had one previous admission a year and a half prior to the present one for delirium tremens. During the month before entry, he was imbibing large quantities of alcohol and eating little. For two months before admission, he noted dark urine and an unsteady gait and, for three weeks, diplopia. No evidence of liver disease had been noted previously. On entry, he showed marked scleral icterus, palmar erythema, spider telangiectasia, moderate ankle edema, and an enlarged nontender liver, which was percussed 7 cm. below the right costal margin. There was no evidence of ascites or splenomegaly. Because of the presence of bilateral external rectus weakness, moderate horizontal and vertical nystagmus, and ataxia, a diagnosis of Wernicke's syndrome was made.

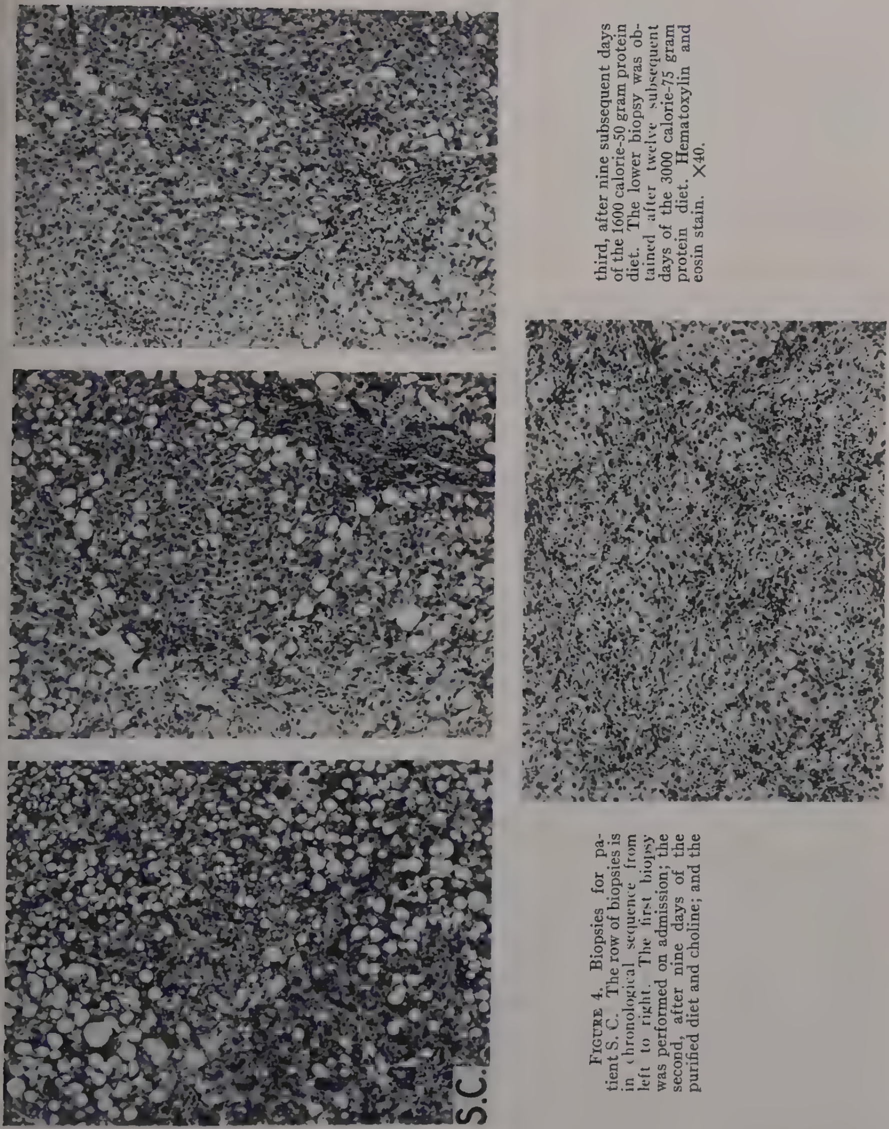

FIGURE 4. Biopsies for patient S. C. The row of biopsies is in chronological sequence from left to right. The first biopsy was performed on admission; the second, after nine days of the purified diet and choline; and the third, after nine subsequent days of the 1600 calorie-50 gram protein diet. The lower biopsy was obtained after twelve subsequent days of the 3000 calorie-75 gram protein diet. Hematoxylin and eosin stain. ×40.

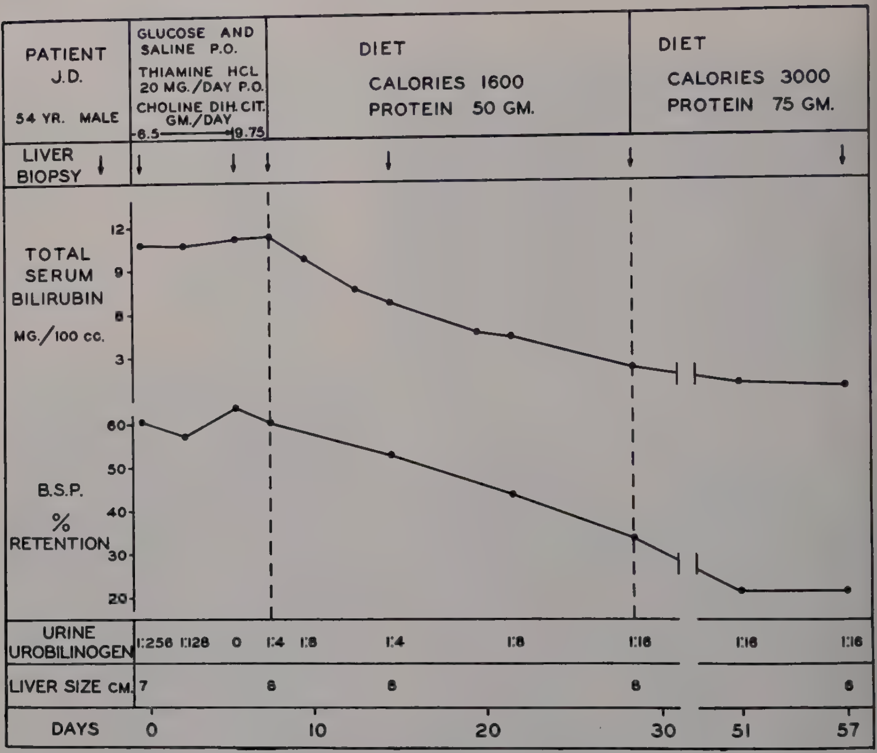

FIGURE 5.

Results (TABLE 1, FIGURE 5): This patient was provided with the purified diet for 7 days. In addition, he received 6.5 gm. of oral choline dihydrogen citrate daily for the first 5 days, 9.75 gm. daily for the last two, 100 mgm. of thiamine hydrochloride orally on the first day, and 20 mg. orally daily over the next 6 days. On this regimen, the serum bilirubin rose slightly but progressively and the bromsulfalein retention remained about the same, although the large amount of dye in the control specimens may have invalidated this measurement to some degree. The urine urobilinogen, however, dropped rather precipitously over this period and, at one time, was thought to be absent. Since stools were all brown, this absence could not be attributed to intrahepatic block of bilirubin excretion. It is noteworthy that the patient had profuse watery diarrhea while on the purified diet and conceivable that this disturbance interfered with the production of urobilinogen.

For the next 3 weeks, the patient was maintained on a 1600 calorie-50 gm. protein diet and for the four subsequent weeks on a 3000 calorie-75 gm. protein diet. After two days of the 1600 calorie-50 gm. protein diet, the serum bilirubin showed a significant decrease and, after 7 days, the serum bilirubin showed a further decline along with a diminution in bromsulfalein retention. These tests continued to improve till the time of discharge. Liver size was difficult to measure, as the patient's inability to relax frequently prevented detection of the edge. By percussion, however, no definite changes were noted.

On admission, the liver biopsy had a rather large amount of fat and fibrosis and a moderate amount of intracellular and canalicular bile, but only rare polymorphonuclear leucocytes and no definite intracellular hyalin. The cells appeared frazzled, and their borders were difficult to discern. Many nuclei were small and dark. Repeat specimens obtained after 5 and 7 days, respectively, of the purified diet and choline displayed essentially the same abnormalities except for a suggestive decrease in fat and less frazzling of the cells. After 7 days of the 1600 calorie-50 gm. protein diet, there was a definite decrease in fat, but no other changes were detected except for an improved cellular architecture and an increase in the number of binucleated cells. The biopsy taken after 3 weeks of this diet had much less fat and intracellular and canalicular bile. Many binucleated cells were observed at this time. The last biopsy specimen, which was secured after 4 weeks of the 3000 calorie-75 gm. protein diet, contained only a minimal amount of fat but considerably more fibrous tissue than on the previous samples.

Case 4. Patient C. W. was a 41-year-old white male chronic alcoholic of 25 years' duration who drank in bouts usually lasting a few months each with intervening periods of abstinence of about 3 months. He had been drinking heavily and eating poorly for 7 months prior to admission. He noted ankle swelling and diarrhea for about 5 days and increasing abdominal girth for 3 days before entry. There was no past history of any of the stigmata of liver disease, but he did have ankle swelling 2 years before, which disappeared when he stopped drinking. Physical examination on admission disclosed a moderate amount of ascites and ankle edema and a firm smooth nontender liver edge made out with difficulty about 5 cm. below the right costal margin. No icterus, splenomegaly, or spider telangiectasia was found.

Results (TABLE 1, FIGURE 6): This patient was given the purified diet plus 6.5 gm. of choline dihydrogen citrate daily for 5 days, the 1600 calorie-50 gm. protein diet for 10 days, and the 3000 calorie-75 gm. protein diet for 16 days. While he received the purified diet and choline, the serum bilirubin rose and the urine urobilinogen remained the same. No initial bromsulfalein test was done. After only one day of the 1600 calorie-50 gm. protein diet, the serum bilirubin was significantly reduced and continued to decline over the next 19 days. The bromsulfalein retention and urine urobilinogen, however, showed no change after 5 days of the 1600 calorie-50 gm. protein diet and decreased slightly, if at all, during the remainder of the study. The liver edge was difficult to palpate because of the ascites and the small changes noted are best disregarded.

Only an admission liver biopsy was done. This showed a moderate amount of fat and marked fibrosis and bile duct proliferation. There were small numbers of polymorphonuclear leucocytes and scattered cells with typical intracellular hyalin.

Case 5. Patient H. C. was a 44-year-old white male who drank in sprees for 30 years, but steadily for the 2 years before entry. About 4 months prior to admission, he increased his alcohol intake and for 2 months prior to admission,

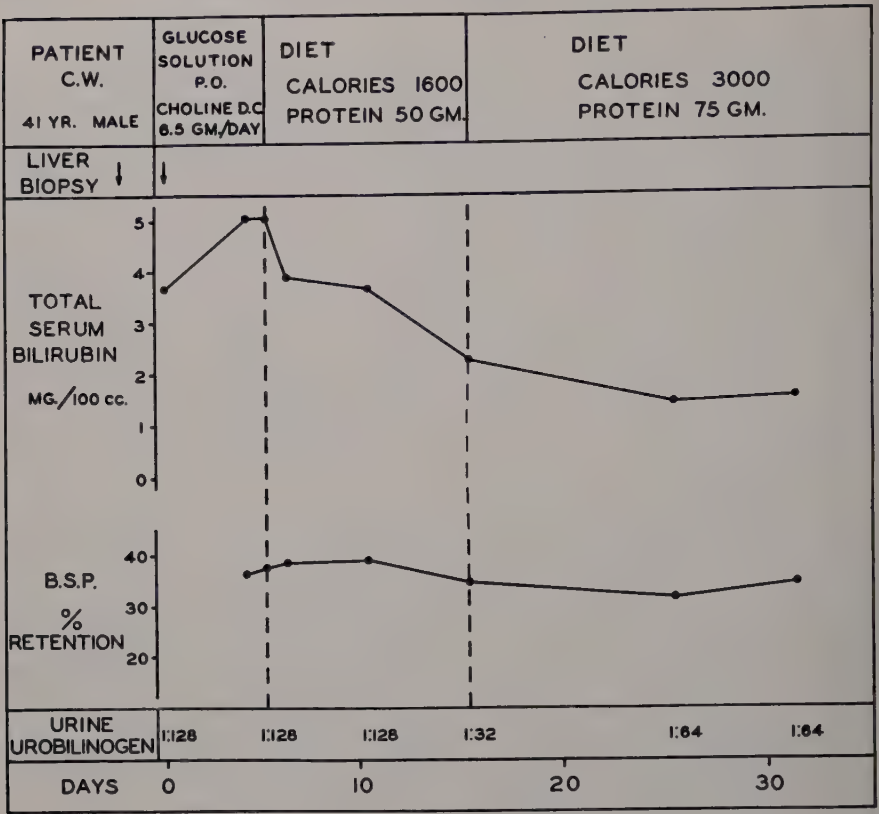

Figure 6.

he was anoretic and ate very little. He lost approximately 50 pounds in weight over the 3 to 4 months before entering the hospital. Dark urine was noted for 3 months, weakness for 1 month, jaundice for 2 weeks, and light stools for 4 to 5 days before entry. There was no previous history of any manifestations of liver disease. Physical examination at the time of admission revealed a slight icteric tint to the skin with marked scleral icterus, a somewhat tender liver with a firm sharp edge extending 11 cm. below the right costal margin, slight gynecomastia, and one spider telangiectasis. No edema, ascites, or splenomegaly was made out.

Results (TABLE 1, FIGURES 7 and 8): This patient was given the purified diet for 4 days, the 1600 calorie-50 gm. protein diet for 10 days, the 3000 calorie-75 gm. protein diet for 10 days, and the regular hospital diet for 28 days. In addition, he received 3.25 gm. of choline dihydrogen citrate daily for the first 24 hospital days. After 4 days of the purified diet and choline, there was a rise in serum bilirubin and urine urobilinogen without a significant change in bromsulfalein retention. After 4 subsequent days on the 1600 calorie-50 gm. protein diet and choline, the serum bilirubin rose even further and the bromsulfalein

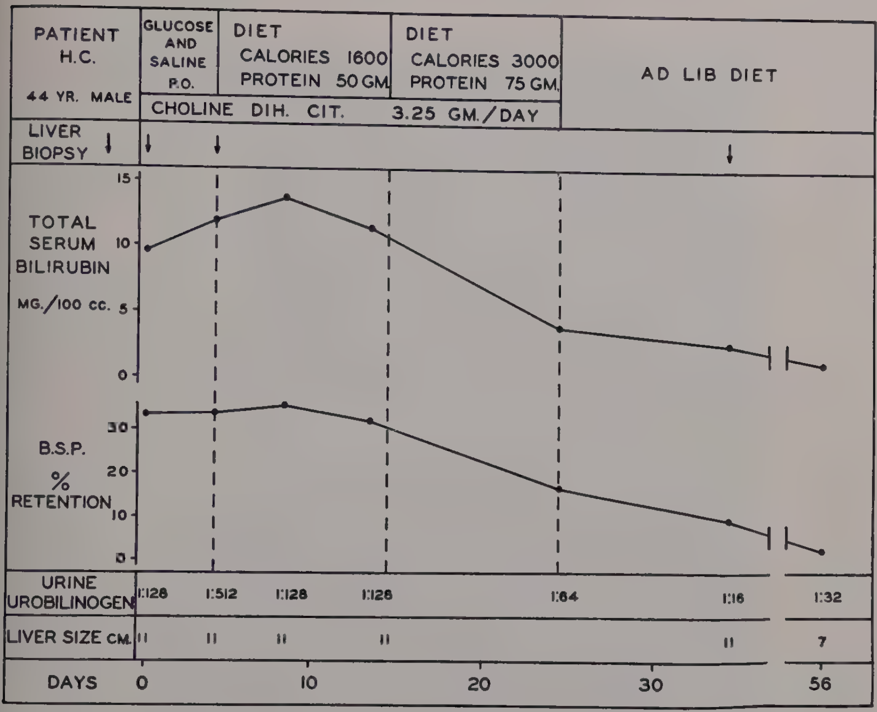

FIGURE 7.

retention remained about the same, while the urine urobilinogen dropped. Six days later, however, the former two tests were decreased and, after that, all three tests gradually improved so that they were within normal limits after 52 days in the hospital. The thymol turbidity and flocculation showed no significant change over the first 14 days but were considerably reduced when measured 24 days after entry.

The initial liver biopsy revealed a small amount of fat with a great deal of intracellular and canalicular bile. There were occasional polymorphonuclear leucocytes and probably a minimal amount of intracellular hyalin. Fibrosis was slight in amount. A second biopsy performed after 4 days of the purified diet and choline showed no discernable change except for a possible slight increase in polymorphonuclear leucocytes. The biopsy taken about a month later, however, disclosed no fat but still an abundance of intracellular and canalicular bile. No intracellular hyalin or polymorphonuclear leucocytic infiltration was seen. In contrast to the first two biopsies, there was marked diffuse fibrosis.

Comment

Of five chronic alcoholics who were provided with the purified diet (glucose and saline) and choline orally for from 4 to 10 days, the active cirrhosis seemed

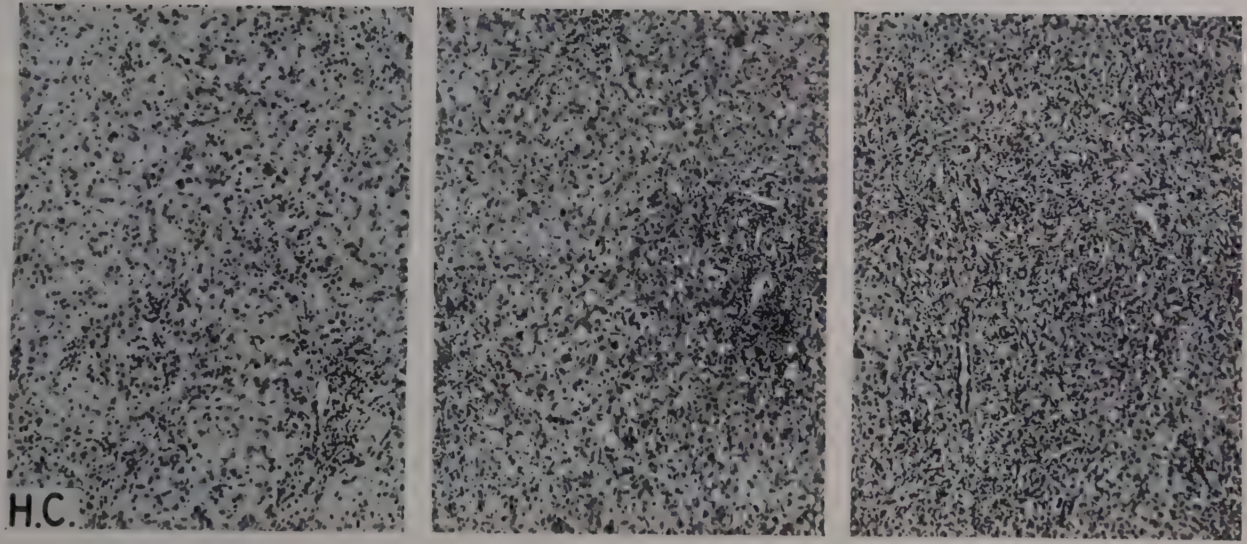

FIGURE 8. Biopsies for patient H. C. The biopsies are in chronological sequence from left to right. The first biopsy was performed on admission; the second, after four days of the purified diet and choline; and the third, after thirty subsequent days of increasingly more adequate diets. Hematoxylin and eosin stain. ×50.

to have improved in 2 and worsened in 3. Although it is possible that the latter three patients received a subtherapeutic quantity of choline, two of them consumed as much or more choline per day as did the two patients who improved. Insufficient gastrointestinal absorption may also have occurred, since two of the patients whose liver disease became worse had diarrhea. Another factor to consider in one of these three patients was a complicating episode of delirium tremens, which may have contributed to the augmentation of the hepatic disturbance. When these three patients were subsequently provided with a more adequate diet, however, the liver disease improved, two of the patients showing a significant decrease in serum bilirubin within one and two days, respectively, after the diet was instituted. This failure to improve on the purified diet and choline with subsequent improvement on a more adequate diet implies that one or more constituents of the more adequate diet was responsible for the mitigation of the active hepatic disturbance and suggests, in turn, that a deficiency of the same one or more factors was important in the development of the liver disease. These results corroborate the findings in a previous study[12] in which the circumstances were similar except that the purified diet was not supplemented with choline. At least in these three patients, the factor does not appear to be choline alone in the amounts given over these brief periods of study. Neither is the factor just calories, as the more adequate diet was isocaloric with the purified diet. What the therapeutic component of the more adequate diet was, therefore, remains unknown.

Even though three of these patients, despite alcohol withdrawal, did not improve until provided with a more adequate diet, the alcohol may still have been an important factor in the development of their liver disease. Its role in this respect might have been to (1) accentuate a nutritional deficiency by increasing the requirement of a nutrient, much as it was thought to do in the studies of Best *et al.*[17] and Klatskin *et al.*[18] using rats; (2) interfere in some way with the utilization of a nutrient; or (3) directly injure the hepatic cell, perhaps being more deleterious to the hepatic cell conditioned by malnutrition.

Improvement in the active cirrhosis did occur in two of the five patients during the period of the purified diet and choline. That choline may have been the therapeutic agent is suggested by the results of the previous study in which the active cirrhosis of three chronic alcoholics did not improve on the purified diet without choline but did improve rapidly on an adequate diet. Lack of adequate control studies, however, prevents this conclusion, as it is quite possible that the liver disease of the patients in the previous study was related to different etiological factors. Indeed, evidence that more than one important factor may be involved in the treatment and possibly the development of the liver disease of alcoholics is provided in the marked difference of response of the liver disease among the five patients in the present study while on essentially the same regimens.

Qualitative differences in the liver disease of the patients who responded favorably and those who did not may account for this discrepancy in response but are difficult to define. With respect to liver function, the only consistent difference was the higher serum bilirubin in the three patients whose liver function grew worse during the purified diet and choline period. With regard to hepatic histology, which was difficult to assess, the two patients who improved during the purified diet and choline period had a larger amount of hepatic fat than the others. In fact, in the one patient who improved the more dramatically, the marked degree of fatty change was essentially the only hepatic lesion noted. The hepatic fat of these two patients, moreover, appeared to decrease during the purified diet and choline period, but a similar change was also noted in one of the patients whose liver disease simultaneously became worse. Thus, hepatic function did not appear to be related only to the fatty lesion, and other elements of the pathological picture, as fibrosis, intracellular hyalin, and necrosis require consideration. In support of the view that fat is not the only histological lesion to consider was the marked acute hepatic dysfunction present in one patient whose liver biopsy showed only small quantities of fat. It is possible that the response of these patients to various dietary regimens is related to the type of hepatic histological abnormalities present, but the small number of patients in this group forbids any rational attempt at correlation.

One striking feature of the histological studies, which were done serially in four patients, was the apparent increase in fibrosis associated with recovery. Although this change may have resulted from the disappearance of fat and condensation of the remaining tissue, the marked increase in fibrous tissue argued for a genuine progression of the fibrosis. To support this impression was a decided increase in fibrosis seen in one of the patients who initially had only a minimal amount of fat. Although the development of fibrosis could not be related positively to any histological feature, there were two bits of evidence against its relation to fat: (1) The patient with the most hepatic fat developed the least fibrosis; and (2) the patient with minimal fat developed pronounced fibrosis. What gave rise to the fibrosis, if real, therefore, cannot be stated from these studies.

Although not all of the active cirrhosis of chronic alcoholics may be on the same etiological basis, in the great majority of these patients, in our experience, this active hepatic disease will improve rapidly following hospitalization of the patient and consumption of even meager diets. In the present study, the active cirrhosis improved in two patients who received merely glucose, saline, and choline, and in three patients who were given only 1600 calories and 50 gm. of protein daily. From our clinical studies, therefore, we believe that effective dietary therapy for the active cirrhosis of the alcoholic is the withdrawal of alcohol and the provision of an adequate diet.

Summary and Conclusions

(1) In a previous study, it was shown that the active cirrhosis of three chronic alcoholics failed to improve when these patients were provided with a purified diet (glucose and minerals), but subsequently improved when given an adequate diet for a similar period. These results suggest that one or more constituents of the adequate diet was responsible for this improvement.

(2) In the present study, five similar patients were similarly followed except for the addition of choline to the purified diet. In three of the patients, choline did not appear to be therapeutic. In the other two patients, choline may have had a beneficial effect.

(3) It is concluded that choline alone in the amounts and for the duration given is not therapeutic for the active liver disease of all alcoholics. Whether choline is of definite therapeutic benefit in some cannot be concluded from the data presented.

(4) The marked difference in response of these patients to similar regimens suggests that the liver disease of the alcoholic may be related to more than one etiological factor.

Acknowledgments

The authors are grateful to Dr. G. Kenneth Mallory, the Mallory Institute of Pathology, Boston City Hospital, for reviewing the histological sections; to Miss Lillian Leavitt of the same institution for preparing the biopsy specimens; to Miss Virginia Olsson for technical assistance; and to Mrs. J. Spencer Rochefort and Miss Evelyn Antonaros for preparing and calculating the diets used.

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Discussion of the Paper

Doctor Harold Blumberg, Endo Products Inc., New York: This investigation by Doctor Davidson and Doctor Phillips is certainly very interesting and valuable. Theoretically, of course, the value of choline in alcoholic fatty cirrhosis might be tested better perhaps by a somewhat different type of experiment, although it is appreciated that this might be difficult to do in the clinic. Choline by itself, or with glucose, cannot be expected to constitute complete therapy. In addition to removal of excess fat there must be regeneration of hepatic parenchyma, and the latter requires protein. Choline cannot provide this necessary protein. It has been suggested only as an adjuvant in the high protein therapy of cirrhosis. There should be two groups of patients, both receiving an adequate amount of protein or amino acids, but not unusually high in methionine. One of the groups should, in addition, receive choline, and the possible value of choline should be gauged by the rapidity and extent of the improvement as compared with the noncholine group.

In 1944 Doctor Russakoff and I reported a partial approach toward this method. Included in a small series of cirrhotic patients with ascites were three who showed no significant improvement after two weeks on a complete diet adequate in protein (protein, 125–200 gm.; carbohydrate, 250–300 gm.; fat, 50–60 gm.). Choline chloride was then added to the regimen in dosages of 3–6 gm. per day. Marked clinical improvement, with decrease in ascites, occurred in all three patients within a week following the start of choline therapy. The coincidence of clinical improvement and choline administration suggested that choline might have therapeutic value.

In our early experiments on fatty cirrhosis in rats, it appeared that the most extensive fibrosis was observed in animals that, after a long period on a high fat and low protein diet, were given additional protein in alternating periods in order to permit some recovery of weight and to prolong survival. Incidentally, the alternation of injury and repair may be somewhat analogous to the pathological history of alcoholic cirrhosis in humans. This was the reason for my previous suggestion that, after this type of liver injury, the high protein therapy not only produces regeneration of normal liver cells, but also may promote the full development of the incipient fibrosis in the areas where the cells have already been badly damaged. Therefore, it is interesting, in this connection, that Doctor Davidson and Doctor Phillips have found that, with complete dietary therapy, their patients showed not only clinical improvement and decreased liver fat, but also an increase in fibrosis in the liver biopsies.

THE PATHOLOGY OF DIETARY LIVER NECROSIS—A PRELIMINARY REPORT

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U. S. Public Health Service, National Institutes of Health, Bethesda, Md.

Hepatic necrosis induced by dietary means, although originally observed in work designed to throw light on cirrhotic processes, has become recognized as a mechanism of great significance in its own right and has been much studied during the last decade. Methods for producing liver necrosis in rats have been well established, with deficient protein-amino-acid fractions of the diet the common denominator of the several dietary means employed.[1, 2, 3, 4, 5]

The necrosis is, in the last analysis, the end result of an underlying degenerative process. Schwarz[6] has introduced the term *dietary necrotic liver degeneration* to emphasize the degeneration as the primary phenomenon. Although much information has been gained with regard to substances which will prevent the necrosis from occurring, still the basis nature of the degenerative process behind it is as yet little understood. In various reports, necrosis has been observed anywhere from the 30th to the 90th day or later following the placing of animals on a suitable dietary regime, with death of the animals usually following the necrosis promptly.

In the current investigations, the "yeast" method of Schwarz was the provocative dietary, procedure a special type of Torula yeast providing the protein in a diet complete in most other respects. This diet has been regarded as being primarily deficient in respect to vitamin E and "Factor 3." From the standpoint of the suitability for histologic study of livers of animals administered this diet, there are certain great advantages, which stem from the consistently high percentage (90–100 per cent) of animals dying with liver necrosis during the span of the 35th to the 50th day, providing many early lesions.

In comparing in a general way the lesions of these animals with those described by other writers, using other but not entirely different diets, it is possible to make the general statement that the lesions are in every way so similar as to indicate that the problem, the nature of the underlying degeneration, is the same. Differences are those of degree and time, but not of general character.

In addition to changes in the liver in the animals studied, other changes, especially in the kidney and in the fore-stomach have been seen, the latter being of the character known to occur in vitamin E deficiency. Although not considered here in detail, they are added evidence of the deficient quality of the diet used.

Livers of more than 400 animals drawn from the experiments of Schwarz have been studied, presenting a broad view of the field. The problem is complex, and the picture is complex. This incomplete report indicates certain lines of investigation of promise for the future, and cautions against unwarranted inferences. In general, findings are in harmony with those of other investigators. Interpretations may differ.

An experiment was undertaken in which animals were killed at predetermined intervals after being on the basic diet 24, 27, 30, and 34 days, a total of 30 animals being used. Frank necrosis was seen in two animals killed on the 34th day. All livers were sectioned and studied for fat and glycogen.

The results paralleled those of Abell, Beveridge, and Fisher,[7] who employed similar procedures. The glycogen was normal in all animals except four killed on the 34th day. The two which showed early necrosis showed no glycogen. In the two others, it was diminished, being found largely in portal areas. There were no definite abnormal fat deposits except in the two animals with early necrosis, in which droplets of moderate size were distributed in moderate numbers throughout the liver. The progressive increase in fat seen by Abell[7] was not observed in the shorter elapsed time of this experiment.

Perhaps the most significant finding of this experiment is the essentially normal histologic appearance of the liver almost up to the point of appearance of the necrosis. It seems important to emphasize the fact that, although the deficiency will routinely kill animals at about the 45th day, there is little histologic alteration in evidence almost up to the point at which necrosis appears.

Types of Liver Cell Degeneration

In the animals dying with or without frank necrosis, two distinctive types of changes in the liver cells had been observed.

The first of these is diffuse karyolysis and karyorrhexis of the liver cell nuclei with relatively little change in the cytoplasm of the liver cells (FIGURE 1). Severe degrees of karolysis have been observed by others, but in the animals dying from the 32 to 35th day, this has been a striking and fairly consistent feature. Livers having areas of necrosis in one lobe will exhibit marked degrees of karyolysis in other lobes in which no actual necrosis appears.

An important aspects of the extensive karyolysis lies in the question of whether, in these animals, the deficiency affects primarily the nucleus of the cells, or whether one is dealing with a deficiency in substances essential to formation of nuclear protein, or essential to nuclear metabolism. The strong impression is that the injury to the liver cell in these animals is a very different thing from the injury due to carbon tetrachloride or other chemical substance, in which the cytoplasm of the cell must necessarily absorb quantities of the chemical irritant.

The sequence of the karyorrhexis-karyolysis process appears to be roughly as follows:

(1) A rearrangement of the nuclear chromatin;

(2) A diminution in size of the nucleus;

(3) Dissolution of the nuclear capsule;

(4) Contraction of the volume of the nucleus as a whole, yet with outlines preserved.

Following dissolution of the nuclear membrane, the nuclear contents do not fuse with the cytoplasm, but the nuclear form is retained for a considerable period of time. Many nuclei will also shrink to become pyknotic, but this type of degeneration appears to follow later.

In cells showing the karyolytic process in its earliest phase, the cytoplasm

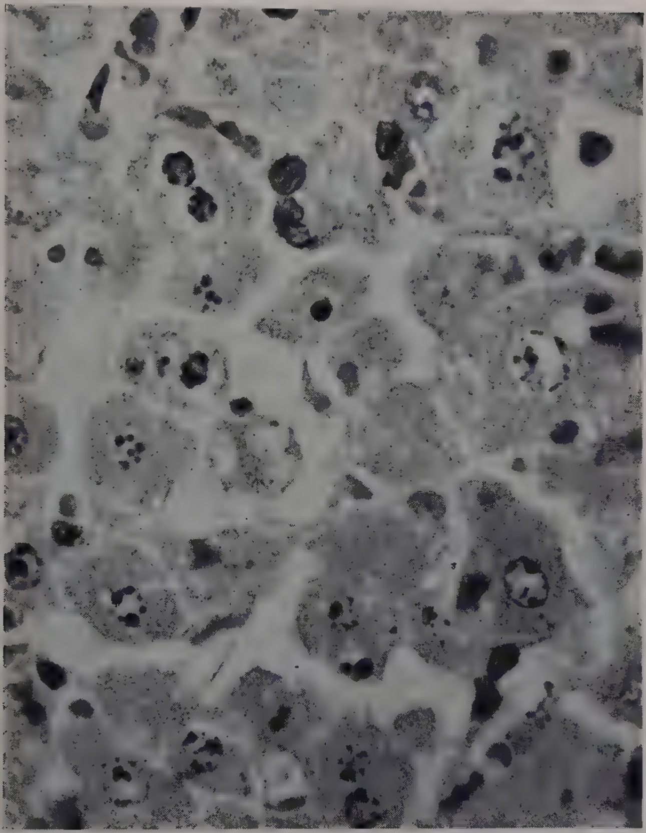

FIGURE 1. Diffuse karyolysis and karyorrhexis of liver cell nuclei during acute phase.

may often appear normal or nearly so. In glycogen and fat stained sections, it is possible to find many cells containing glycogen, but no fat, yet illustrating the beginnings of the karyolysis. However, it is clear that these cells do lose their glycogen in a short time, probably a matter of a few hours, once the process is started, and likewise acquire cytoplasm lipoids at the same time. Once the karyolysis is well established, the cytoplasm often appears frayed out, somewhat watery, perhaps increased in over-all volume. But the point to be made is that the nuclear degeneration often appears to precede the cytoplasmic.

It is dangerous to draw too much inference from this histologic change. The liver may appear normal on the 33rd day and necrotic on the 34th. It is obvious that the histologic evidence of liver cell injury is only a partial measure of what goes on.

Another curious finding is the occurrence of numerous mitotic figures in liver cell nuclei in occasional animals, which are undergoing this degenerative process. The mitoses occur at random anywhere and are far more numerous than ever seen in livers undergoing regeneration. Evidently there is a sudden crop of them, as though the primitive mechanism of reproduction as a protective against destruction has been evoked. Although this phenomenon has been seen in only about 2 per cent of all the livers examined, it is too striking to go without mention, and is further evidence of the profundity of the alterations in the liver cell.

A *second* type of degenerative change has been observed in many animals, often in those showing varying degrees of diffuse karyolysis, but not conjointly within the same cell. This is seen first as fine refractile eosinophilic granules evenly distributed in the cytoplasm. These are quite small, of the order of 1–2 μ in diameter. When cells containing these bodies become separated from neighboring cells, as they often do, the granules become larger, and finally undergo calcification (FIGURE 2).

The various stages of formation can be followed through various animals, although in a given animal the changes throughout are essentially of the same age and degree. The cell form is often retained, perhaps supported by the calcific bodies. The nucleus is unaltered at first, later fading out of existence without undergoing karyorrhectic changes.

This sequence of events has been seen to best advantage in animals dying or

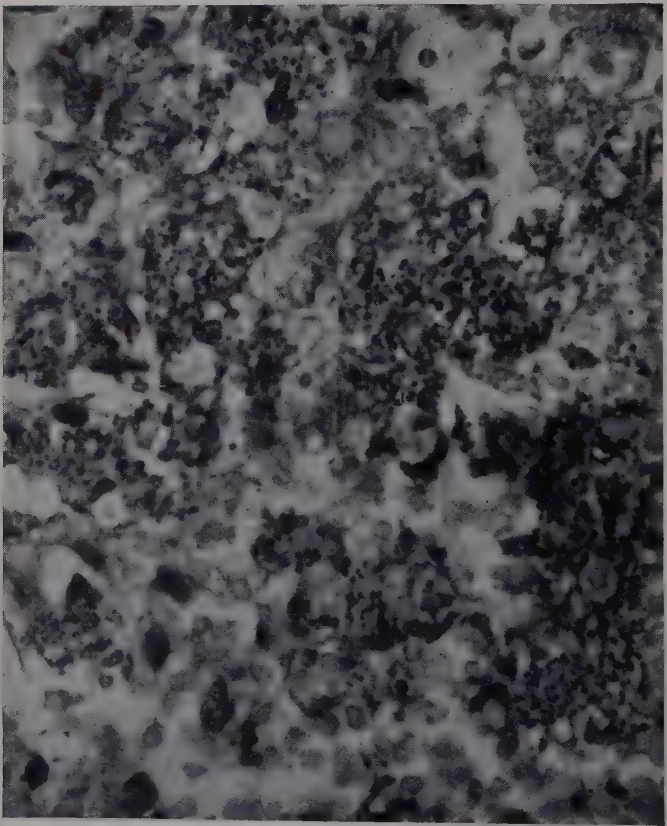

FIGURE 2. Cytoplasmic granules in degenerating liver cells, advanced stage with calcification.

killed in both early and subacute stages of the process, as well as in a few animals which had been fed protective substances after the period of potential necrosis was well under way. It seems probable that the eosinophilic granulates of the cytoplasm become calcified only infrequently, the determining factors being unknown.

Up to the present time, the exact relation of this type of degenerative process to the other is not understood. This type of lesion had not been seen too frequently to the point of calcification of the bodies, but it has been possible to demonstrate the early cytoplasmic change in the liver cell many times. The mechanism appears to be quite different from that of diffuse karyolysis. Here the nucleus is unaltered until after the process of granular body formation is well developed. That the granules may develop from altered mitochondria is a strong possibility, proof of which is as yet wanting.

The Circulatory Factors

Himsworth and his associates[3, 4] have stressed the circulatory factor in liver necrosis, especially with respect to the distribution of lesions. The distribution of the areas of necrosis in these animals has been essentially the same as that observed by all other investigators: more common at the free margins of the lobes; more common where these are in contact with other viscera; more common in lobes which are thin, than thick. Himsworth went to great lengths to show that a failure of splenic and portal blood to mix promptly might be responsible for differences; in other words, that the differences might be related to a condition when one lobe receives more blood directly from the intestines, while another receives a majority of its venous blood from the spleen. While not attempting to repeat all of Himsworth's detailed studies relative to this problem, India ink was injected into some thirty animals on the 33rd day, at a time when liver necrosis was incipient. The injections were made in the spleen, and the animals killed a few hours later. As nearly as could be judged from gross observation of blackening of the liver immediately following the injection, and from microscopic sections, the ink was evenly distributed to all lobes, and promptly taken up by the Kupffer cells. Only minor differences of lobular distribution were observable, such as might be attributed to a temporary increase or decrease of flow to a given area. Several animals showed early stages of degeneration, in which the ink was deposited as in normal areas. Three animals with early necrosis showed absence of ink from some necrotic areas, but by no means all. The only conclusion to be drawn is that any alteration in circulation occurs after, and not before, the occurrence of necrosis.

A second consideration is the distribution of necrotic areas with respect to the liver lobule. Experience has been that there is no absolute rule with respect to the occurrence of areas of degeneration or necrosis, although there are preponderances:

(1) There is no focal necrosis. Areas of necrois are invariably larger than the individual lobule.

(2) The usual area of frank necrosis involves an area of the liver without respect to its main blood supply. Exceptions occur, however, in which the

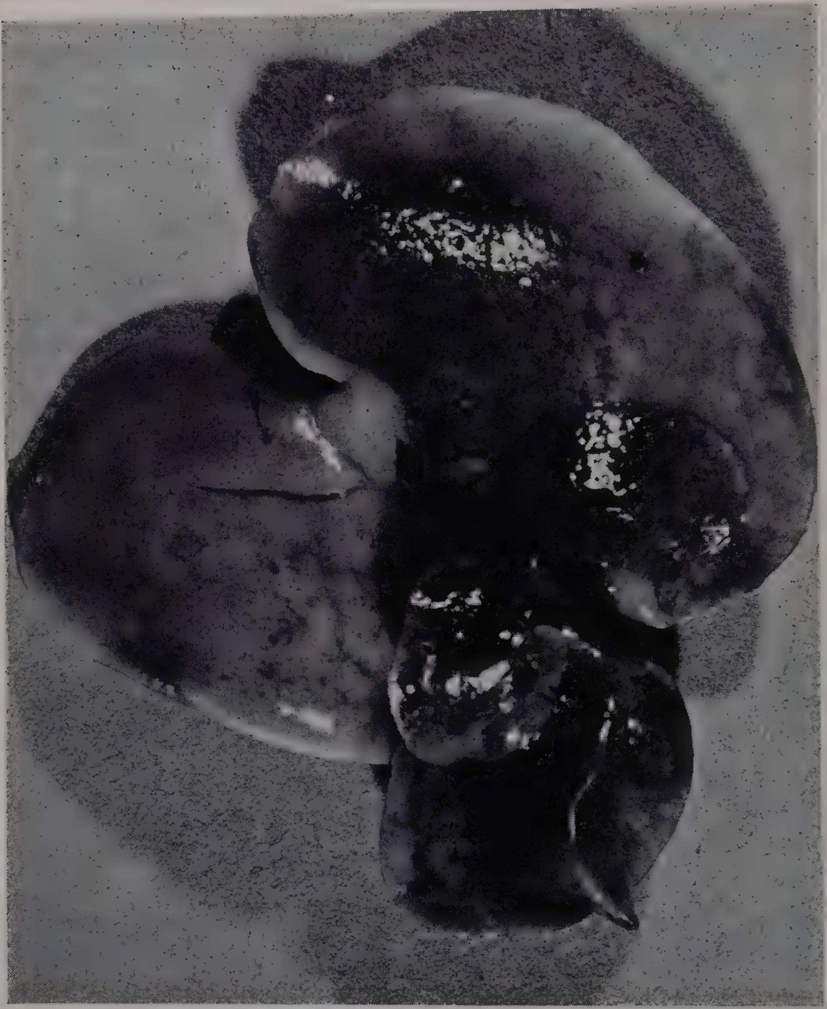

FIGURE 3. Gross appearance of liver of rat dying with acute necrosis.

necrotic areas have an irregular ramifying serpentine pattern analogous to the branchings of *efferent veins*.

(3) There is commonly a narrow zone of preserved cells adjacent to portal areas. In frankly necrotic areas, this is extremely narrow, at best. In degenerating areas, a layer 3 to 5 cells thick around portal zones may be normal.

(4) Inversely, there are many examples in which the preponderant areas of degeneration or necrosis, or both, are in a sense centrolobular. This is never, however, a discrete central zone, but a broad area making up most of the lobule.

It becomes clear that the circulatory arrangement of the liver does have an effect on the distribution of the areas of necrosis, in that the more immediately nourished portal cells suffer the degenerative changes slightly less.

Once necrosis has occurred, the resulting effects on the circulation in the necrotic and neighboring areas seems to be quite capricious. In some examples, the efferent blood flow is partly or completely shut off, which means that, in addition to the original dietary necrosis, the added necrotizing factor of anoxemia may produce still further change. In most cases, the animal dies too quickly for this to be histologically clear-cut.

A second result of the necrosis is the "hemorrhage" which is seen frequently. For the most part, this so-called hemorrhage is an accumulation of blood within sinusoids at the margins of necrotic areas, rather than extravasation outside the normal channels. Another type, however, consists of pools around efferent veins, suggesting some degree of back-tracking of blood from efferent veins into necrotic areas. Again, these accumulations are within sinusoids, and true hemorrhage (*i.e.*, the escape of blood from vascular pathways) probably does not occur.

It is impossible to estimate the degree to which simple congestion may operate to provoke frank necrosis. Yet when one sees frequently the state of degeneration which has occurred in more "normal" areas in these necrotic livers, it is easily supposed that an extremely slight alteration in blood flow might operate to initiate or to advance the formation of a large area of necrosis. Under the circumstances, it hardly seems necessary to conceive a gross alteration or variation in the hepatic circulation to explain the change from degeneration to necrosis.

Delayed Liver Necrosis

In the course of this work, many animals have been studied which have been given, in addition to the basic diet, supplementary substances at levels which have been partially effective in preventing the development of necrosis, *i.e.*, only a certain number of the animals have actually died from necrosis. Here the picture may be different in several ways from that of animals dying early in the disease.

(1) The picture of diffuse karyolysis is shown to better advantage or to a greater degree in animals dying at about the 50th day than in animals dying on the 35th day on the basic diet. This seems to support the idea that the diffuse karyolysis is truly a histologic expression of the underlying degenerative status. A longer period of time allows a greater degree of degenerative change to become manifest. Animals given slightly insufficient amounts of protective proteins and surviving longer periods of time seem to illustrate degenerative processes to better advantage than animals dying in the earlier acute phase.

2. Abell[7] speaks of submassive and recurrent submassive necrosis. These changes are similar to those we have observed in some animals dying at later time intervals. He illustrates the phenomenon of numerous mitotic figures in this "recurrent-submassive" variety. In our animals, histologic evidence of recurrent necrosis has been seen (1) where a delicate balance between death and survival was maintained, or (2) when scars and regenerated areas obviously resulted from previous injury. Schwarz[5] has observed grossly such scars in 24 per cent of 1600 unselected animals dying of liver necrosis.

It is readily possible to prevent the necrosis by suitable dietary means at any time prior to its actual occurrence. In a number of animals so protected, and killed later, the appearance of the liver has usually been that of the normal animal. In a few, scarring and regeneration have appeared. Except for these evidences of repair and regeneration of frankly necrotic areas, no changes have been seen. Thus, present indications are that the degenerative changes are reversible to a substantial degree, although the limits have yet to be defined.

Cirrhosis has never been observed in these animals on the necrogenic diet, nor has the picture of the fatty liver of choline deficiency been seen.

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Discussion of the Paper

Doctor Hans Popper, *Hektoen Institute, Chicago, Ill.:* I should like to congratulate Doctor Fite on a thorough and much needed investigation upon the histologic changes in acute hepatic necrosis. It is worth emphasizing that histologically insignificant changes precede the explosive, sudden breakdown of the liver cells and that nuclear changes usher in the lesion. The eosinophilic necrosis described is also interesting from the point of view of the human pathology where it seems to differ in appearance with different etiologic factors. In viral hepatitis, it usually takes the form of a diffuse homogenization of the cytoplasm (similar to the so-called Councilman bodies) whereas in toxic conditions, granular coagulation necrosis of the cytoplasm of the character of Mallory bodies is more common. This raises the question as to the general behavior of the cytoplasmic basophilia, supposedly due to the presence of pentose nucleic acid in nutritional necrosis. I also should like to ask about possible alterations of the reticulum framework in the animals studied. In most instances of human hepatic necrosis presumably produced by either viruses or chemical toxins, it remains intact, whereas it is broken in some types of toxic hepatitis and as the result of ischemia. Obviously, the reticulum changes would be of great importance in recovery stages from such necrosis.

DIETARY METHODS FOR INDUCTION OF NECROTIC LIVER DEGENERATION

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Necrotic liver degeneration is readily induced in young rats by the dietary control of two factors, sulfur-containing amino acids and Vitamin E. I am aware that the modern interpretation requires at least three factors, cystine, vitamin E, and Factor 3; but I shall leave their elucidation to Doctor Klaus Schwarz. Doctor Daft has given an interesting account of the historical sequence of factors in nutritional liver injuries. The preventive effect of vitamin E in liver necrosis was observed by Schwarz (1944), György (1947), György and Goldblatt (1949), Hove, Copeland, and Salmon (1949), and others; nevertheless, it was Schwarz (1944, 1949), and Himsworth and Lindan (1949), who recognized the importance, in experiments of this type, of restricting initial stores of vitamin E in the organs and tissues of the rat. This factor of controlling the initial stores of vitamin E appears to me to be a vital one.

Diets. While yeast diets are commonly employed, a high incidence of the disorder may be observed with crude casein as the source of protein (Goettsch, 1951), which was the protein used by the original investigators. The composition of the diets is given in TABLE 1. Diet 1 is typical of diets used in Vitamin E experiments. Diet 2 contains the same ingredients as the low-protein diets 3 to 6. When α-tocopherol was added, it met the minimum protein requirement for growth and reproduction in several generations of rats.

Supplements were introduced at the following concentrations: DL-methionine, 0.1 to 0.3 per cent; L-cystine, 0.1 to 0.3 per cent; and dl-α-tocopherol acetate, 2 mgm. per rat per week.

Procedure. Wistar or Sprague-Dawley rats were used. Breeding rats were reared and bred on the low-vitamin-E but adequate diets 1 and 2, which were equally suitable for the preparation of young. To permit the birth of young, the rats were given 10 mgm. of α-tocopherol at the beginning of the first gestation, 20 mgm. at the second, and 30 mgm. at the third. The young did not develop muscular dystrophy at the end of lactation. In this way, young with initial low stores of vitamin E were obtained.

These young rats were given diets 3 to 6 from the 21st day at which time the body weight was approximately 35 gm.

Results and Discussion

Rats with the low-casein diets grew poorly. Nearly all of them, about 90 per cent, died suddenly after 5–40 days. In recent trials, the incidence reached 100 per cent and none of the rats survived for longer than 20 days. Liver necrotic degeneration was observed grossly and confirmed by Doctors Koppisch and Izquierdo of the Department of Pathology of the University of Puerto Rico School of Medicine by microscopic examination. Some of the rats presented early lesions of muscular dystrophy.

TABLE 1

COMPOSITION OF EXPERIMENTAL VITAMIN-E-LOW DIETS

Constituents*	Diets for breeding rats†		Isocaloric low-protein diets			
	1	2	3	4	5	6
Crude casein	26.6	20.0	7.5	8.3	10.0	11.4
Yeast, brewers' type	16.6	—	—	—	—	—
Cornstarch	33.3	68.0	86.5	79.7	64.0	48.0
Salt mixture‡	3.4	4.0	4.0	4.0	4.0	4.6
Lard	18.3	6.0	—	6.0	20.0	34.0
Cod liver oil	1.8	2.0	2.0	2.0	2.0	2.0

* Synthetic vitamins added as supplements to each kilogram of diets of 2 to 6: thiamine HCl, 5 mgm.; pyridoxine HCl, 5 mgm.; riboflavin, 10 mgm.; niacin, 20 mgm.; Vitamin K, 5 mgm.; PABA, 10 mgm.; Ca pantothenate, 50 mgm.; folic acid, 4 mgm.; and biotin, 0.4 mgm.

† Ten to 30 mgm. dl-α-tocopherol acetate given at beginning of each gestation.

‡ Modified Hawk-Oser (1931) salt mixture, with $CaCO_3$ substituted for Ca citrate.

After some weeks, the survivors ceased to grow and acquired the chronic muscular dystrophy that was described by Einarson and Ringsted (1938) in low-E rats. A few that survived 90 days were bred. They manifested the typical sterility of vitamin E deprivation (Evans and Bishop, 1923; Mason, 1926). At death, these rats presented normal liver. The reason for this result is unknown. It may be that the wasting away of muscle releases sufficient of the sulfur-containing amino acids to protect the liver.

Influence of lard and cod liver oil. Lard and cod liver oil are important variables in the induction of vitamin E sterility in rats.

The incidence of liver necrotic degeneration was not influenced by variations from 0 to 34 per cent in the lard content of the isocaloric diets. The results were not altered with three different types of lard, modern processed, old type, or Eastman low E. In other diets, afaxin and viosterol were substituted for the cod liver oil. All of the rats developed fatal hepatic necrosis. It appears that the disease occurs in the absence of cod liver oil and is independent of the lard concentration of the diets, under the given conditions.

Variations in the content of casein. The protective effects of high casein diets are well known. The crude casein concentration was varied from 5.8 to 14.0 per cent in diets containing 6 per cent lard. All of the rats with the 5.8 per cent diet, 2 of 6 with 11.6 per cent casein, and none of 24 with 14.0 per cent casein acquired liver necrotic degeneration. Chronic muscular dystrophy appeared in all rats that did not develop liver lesions. They survived for 4 to 6 months, presenting severe muscular dystrophy and normal liver at death.

Other sources of protein. Under the given conditions, liver necrotic degeneration has been observed with low-E—low-protein diets (Goettsch, 1948, 1951) in which the source of protein was primary grown American brewers' type yeast (Fleischmann, type 2019), food yeast (*Torula utilis*, number 3, locally grown), polished rice, red kidney beans, or gelatine. Whether these proteins contain the factor 3 of Schwarz (1951) is not known.

Initial size of the low-E rat. Since it has been customary to use rats with an initial body weight of 70–100 gm., young low-E rats were maintained with the low-E—high-casein diet until they weighed 70–100 gm. With increasing initial body weight, there was a decrease in the incidence of liver necrotic degeneration and an increase in the time of survival. This observation confirms that of Lindan and Himsworth (1950).

Initial stores of vitamin E in the rat. There is great variation among rats in their ability to store vitamin E in the tissues and in the requirement of vitamin E for reproduction and for the prevention of muscular dystrophy. In vitamin-E-low rats that were given one single dose of 2 mgm. α-tocopherol on the 15th day of lactation, the incidence of liver necrotic degeneration was reduced 50 per cent.

The discordant results among different laboratories in the induction of liver degeneration presumably have been associated with the amount of α-tocopherol supplied during the pre-experimental period. It was recently observed that some rats, reared and maintained with a commercial ration, underwent resorption gestations when they were given the low-E—high-protein diet from the beginning of gestation. These results suggest an explanation for the induction of liver necrotic degeneration in rats that have not been purposefully prepared to contain low initial stores of vitamin E.

Addition of methionine and cystine. The preventive effect (Weichselbaum, 1935, Daft, Sebrell, and Lillie, 1942) of the sulfur-containing amino acids is well known. Although these experiments were not quantitative, as were those of Schwarz (1952), the addition of 0.3 per cent of either DL-methionine or L-cystine to the low-casein diets prevented the liver necrosis in a high proportion of the rats, under the given conditions. As with other groups of rats, chronic muscular dystrophy and sterility developed in all rats that did not acquire the hepatic degeneration. The livers were normal at death, which occurred after many months. The results appeared to be similar to those of the rats with 11.6 per cent casein diet.

Addition of α-tocopherol. Liver necrotic degeneration was prevented in all rats receiving 2 mgm. α-tocopherol per week, in confirmation of the results of Schwarz (1944), György (1947), and György and Goldblatt (1949). The α-tocopherol not only prevented liver necrotic degeneration but also protected the rats from muscular dystrophy and sterility. When the rats were sacrificed after a survival period of 6–10 months, certain changes were noted in the livers. Thus, while about one fourth appeared normal, one half presented very fatty livers, and one fourth presented diffuse hepatic fibrosis. The incidence of liver injury was highest among rats with the high lard diets. The addition of 0.3 per cent DL-methionine tended to prevent the fatty changes and cirrhosis. The addition of 0.3 per cent L-cystine aggravated the intensity of the lesions, confirming the observations of Best and Huntsman (1932), Tucker and Eckstein (1937), Earle and Victor (1941), and others.

Summary

(1) With low-E—low-crude-casein diets, liver necrotic degeneration appears rapidly in young rats deprived of Vitamin E. Early lesions of muscular dystrophy may occur.

(2) These lesions develop in the absence of cod liver oil and are independent of the lard content of isocaloric diets.

(3) They occur with the following sources of protein: primary grown brewers' type and torula dried yeast, polished rice, red kidney beans, or gelatine.

(4) An increase in the available protein, by the addition of casein, cystine, or methionine, decreases the incidence of liver degeneration and permits the animals to survive for a long period of time. These rats acquire chronic muscular dystrophy and sterility, but at death the liver is normal.

(5) The addition of α-tocopherol invariably prevents liver necrotic degeneration enabling the rat to survive for a long period of time and to reproduce under conditions that are associated with fatty deposition in the liver and cirrhosis. Methionine prevents, and cystine intensifies, fatty infiltration of the liver and diffuse hepatic fibrosis.

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DIETARY HEPATIC NECROSIS IN THE RAT—ABSENCE OF CIRRHOSIS FOLLOWING RECURRENT EPISODES*

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The investigations to be described arose from an interest in the human disease, postnecrotic cirrhosis, and represent an attempt to produce an experimental form of the disease. This type of cirrhosis presents a challenge to the pathologist and to the clinician. The etiologic factor or factors responsible are obscure. The morbid anatomy is variable. The clinical course is, however, fairly constant. Patients with postnecrotic cirrhosis quite uniformly exhibit a gradual but progressive deterioration of hepatic function and the disease ultimately proves fatal. The clinical course and the rate of progression of the disease is subject to great individual variation. We are not convinced that the natural course of cirrhosis of this type can be fundamentally altered by nutritional therapy. Perhaps all such patients are victims of an infectious liver disease, chronic viral hepatitis in the cirrhotic stage. Many investigators believe so. It seems very likely that at least some cases of postnecrotic cirrhosis do follow an attack of viral hepatitis.

There is a similarity between one form of cirrhosis seen in the clinic (the patient who is a victim of chronic alcoholism and consequent malnutrition) and one form of experimental cirrhosis (the rat that is a victim of prolonged fatty infiltration of the liver). The investigator possesses a laboratory method by which he can produce a disorder that resembles in many respects a disease that he encounters in the hospital wards. In attempting the production of an experimental form of postnecrotic cirrhosis, an obvious approach would be to cause chronic viral hepatitis in an animal. This most desirable accomplishment seems quite unobtainable at the present time. Hence, we sought to produce a structural counterpart of this disorder by inducing liver damage through a mechanism that was available. The lack of any etiologic similarity between the method used, experimental dietary necrosis, and viral hepatitis was recognized and appreciated.

In the experimental production of any type of cirrhosis, repeated exposure to the insulting or damaging agent is an essential feature. When carbon tetrachloride is employed for this purpose, the investigator administers the toxic agent twice a week, three times a week, week after week, until he obtains the desired goal. In the case of fatty infiltration, the damaging effects of the fatty cysts proceed continuously night and day in the fashion that Hartroft[1] has demonstrated. Would it be possible to produce a structural counterpart of human postnecrotic cirrhosis if an animal could be forced to undergo repeated episodes of dietary liver necrosis? The following studies were directed toward such a goal. There are two aspects to be reported: (a) the resultant pathologic changes observed after the spontaneous occurrence of repeated attacks of dietary necrosis; (b) the resultant changes noted when one attempts to modify the course of events after one or more episodes of necrosis have occurred.

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* These investigations were sponsored by the Commission on Liver Disease, Armed Forces Epidemiological Board and supported in part by a contract from the Office of the Surgeon General, U. S. Army.

TABLE 1
COMPOSITION OF DIET-BY18

Sucrose	72%
Yeast[1]	18%
Salts[2]	4%
Vitamin powder[3]	1%
A-D Vitamin mixture[4]	0.05%
Lard[5]	4.95%

[1] Dried Baking Yeast; United Yeast Co. Ltd. London.

[2] Salt Mixture No. 2; General Biochemicals, Inc.

[3] Vitamin powder supplies: 25 μg. thiamine, 40 μg. riboflavin, 25 μg. pyrodoxine, 100 μg. ca pantothenate, 100 μg. nicotinic acid, and 25 μg. Vitamin K in the daily allowance (8 gm.) of diet. For easier mixing, the vitamins are prepared with powdered sugar.

[4] A-D Vitamin concentrate*: one part of the concentrate mixed with 7 parts of peanut oil, and used as specified, furnishes 100 units of Vitamin A and 25 units of Vitamin D per 8 gm. of ration.

[5] A special lard, tocopherol content minimal.†

* Kindly made available by Ayerst, McKenna and Harrison, Ltd., New York 16, New York.
† Kindly made available by Hormel Company, Austin, Minnesota.

Experimental Methods Employed

Throughout these experiments, a uniform strain of white laboratory rats* was used. Male rats weighing 50 ± 5 gm. at the start of the experiment were routinely employed except in the initial investigations (described in TABLE 2). The animals were housed in individual cages suspended in racks of a type that provided a continuous sheet of semi-waterproof paper beneath the cages. This arrangement proved convenient, since daily urine tests for bilirubin were necessary. Specimens for testing were secured from small pools of urine that collected beneath each cage. Franklin's[2] method for detecting bilirubin in the urine was employed routinely in these studies. Daily urine tests were begun 20 days after the animals were started on the necrogenic diet.

Diet. The diet employed in the major portion of this study was designated by BY18. The composition is given in TABLE 1. Each rat was allowed 8 gm. per day. Fresh rations were mixed once each week and refrigerated. The animals were fed three times each week. Food consumption was not routinely measured. Free access to water was allowed.

In preliminary investigations, diets of slightly different composition were given as indicated in TABLE 2. The diet designated as H2 was described in a previous report.[3] The diets designated as TY20 and TY35 contained 20 and 35 percent *Torula* yeast† respectively, and differed from the BY18 diet in only two respects. There was a reduction in the sucrose content to 70 and 55 per cent respectively. The vitamin mixture employed did not contain vitamin K. The diet BY18R differed only in that 1 ml. of a liquid resin preparation‡ was added to the 8 gm. ration allowance offered daily to each animal.

Pathological analysis. Animals were autopsied as soon after death as possible. Where the animals were obviously moribund, they were anesthetized and sacrificed. The livers were fixed in a neutral formalin solution. At the

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* Purchased from Holtzman Rat Company, Madison 5, Wisc.
† Supplied by the Sulphite Pulp Manufacturers' Research League, Appleton, Wisc.
‡ RESION R, supplied by the National Drug Co., Philadelphia, Pa.

TABLE 2

INCIDENCE OF POSTNECROTIC SCARRING OF THE LIVER IN RATS DYING OF DIETARY NECROSIS

No. of rats	Sex	Initial weight (± 10%)	Diet*	Survival period (days)	Degree of Scarring		
					Absent	Minimal	Marked
10	M	50 gm.	H2	34–72	5	4	1
18	F	50 gm.	H2	27–74	11	4	3
10	M	100 gm.	BY18	63–116	3	7	—
10	F	100 gm.	BY18	64–144	6	4	—
9	M	100 gm.	TY20	43–137	8	1	—
13	F	100 gm.	TY20	74–202	8	5	—
3	M	100 gm.	TY35	72–106	—	3	—
5	F	100 gm.	TY35	118–180	3	2	—
17	M	50 gm.	BY18 R	25–59	2	10	5
70	M	50 gm.	BY18	28–70	21	32	17

* Diets: H2—18% Baker's yeast, corn starch, and oil (see Text); BY18—18% Baker's yeast, sucrose, and lard (see Text); TY20—20% Torula yeast, sucrose, and lard (see Text); TY35—35% Torula yeast, sucrose, and lard (see Text); BY18 R—1 ml. resin (Resion) added to daily ration (see Text).

completion of an experiment, all specimens were graded as to the degree of scarring. Appropriate sections were then removed for histologic study. A massive hemorrhagic necrosis of the liver was the cause of death in all rats except as described subsequently (TABLES 4 and 5). This lesion can be dedetected by gross inspection. The presence of scarring indicative of one or more previous episodes of submassive necrosis can also be detected by careful gross inspection. The scarring was graded as *minimal* if the left lateral lobe alone was involved and as *marked* if one or more additional lobes revealed the characteristic pits or furrows. Microscopic sections were prepared by standard procedures. Hematoxylin eosin and azocarmine stains were used most frequently. In a few instances, plastic corrosion preparations of the intrahepatic veins were made by injecting colored vinyl acetate solutions into the portal and hepatic veins.

RESULTS

The presence of depressed scars in the liver of a rat dying of dietary liver necrosis is an indication that the animal has undergone one or more previous episodes of liver cell degeneration. The sequence of events that leads to the development of such lesions has been well described by Himsworth[4] and by Abell, Beveridge, and Fisher.[5]

The incidence of postnecrotic scarring of the liver in the present series of experiments is shown in TABLE 2. Various types of necrogenic diets were employed. A low incidence was encountered until young growing male rats were employed. This was first observed during a study in which the diet BY18R was used. The addition of the resin compound had no influence on the course of the disease or the resultant pathology (the 17 controls for this particular experiment are included among 70 males in TABLE 2). A high incidence of re-

TABLE 3

INCIDENCE OF POSTNECROTIC SCARRING AND CORRELATION WITH BILIRUBINURIA IN RATS DYING OF DIETARY HEPATIC NECROSIS

No. of rats*	Per cent of total	Survival range	Incidence of bilirubinuria		Postnecrotic scarring
			Intermittent	Terminal	
21	30%	28–65 Days	0/21	9/21	Absent
32	45%	28–58 Days	4/32	18/32	Minimal
17	25%	34–70 Days	13/17	14/17	Marked
70	100%				

* Males, initial weight = 50 ± 5 gm.

current episodes of dietary necrosis, as judged by postnecrotic scarring, had occurred both in the control and in the resin-treated group. Thereafter, 50 gm. male animals were employed and recurrent necrosis, as a spontaneous feature of the disease, was commonly encountered.

We had been unable to detect, with any appreciable degree of accuracy, the occurrence of nonfatal episodes of dietary necrosis by observing the behavior of the animal or by measuring food intake or body temperature. Serial studies of the leukocyte count or the level of the pseudo-cholinesterase content of the plasma failed to furnish a satisfactory clue.[6] The association of bilirubinuria with episodes of recurrent necrosis was cited by Himsworth and Glynn.[7] We had observed bilirubin in the urine of some rats just before they died of massive hepatic necrosis. A systematic study of the occurrence of bilirubinuria during the life of such animals was undertaken. Daily urine tests were performed beginning twenty days after the start of the diet. The results obtained in 70 male rats are shown in TABLE 3.

The degree of postnecrotic scarring was graded as "marked" in 17 rats of this group of 70 young growing males. Recurrent episodes of dietary necrosis had therefore occurred as a spontaneous feature. As may be seen in FIGURE 1, intermittent bilirubinuria occurred in 13 of the 17 animals. We believe that the transitory appearance of bilirubin in the urine coincided with some nonfatal episodes of hepatic necrosis. It is apparent that this method of serial testing failed to detect all instances of recurrent necrosis. For example, bilirubinuria was not detected in rat number 16 (FIGURE 1) at any time, yet postnecrotic scarring of the liver was marked. That bilirubinuria does not constantly accompany fatal hemorrhagic liver necrosis in the rat is also apparent from the data in TABLE 3. Despite the obvious limitations, the simple procedure of daily urine testing did furnish a means of following the natural course of the disease. It also offered a means by which one might alter the course of the disorder by changing the diet *after* a recognizable episode of necrosis had once occurred. This phase of the study will be discussed later.

The gross appearance of the liver of an animal exhibiting a moderately severe degree of postnecrotic scarring (rat number 17, FIGURE 1) is shown in FIGURE 2. It is a representative sample of this group of 17 rats. Sections were prepared for histologic study from each of the livers in this group. Areas of various lobes exhibiting gross distortion were sectioned in different planes. The pathologic picture was one of acute hemorrhagic liver necrosis (this was the cause

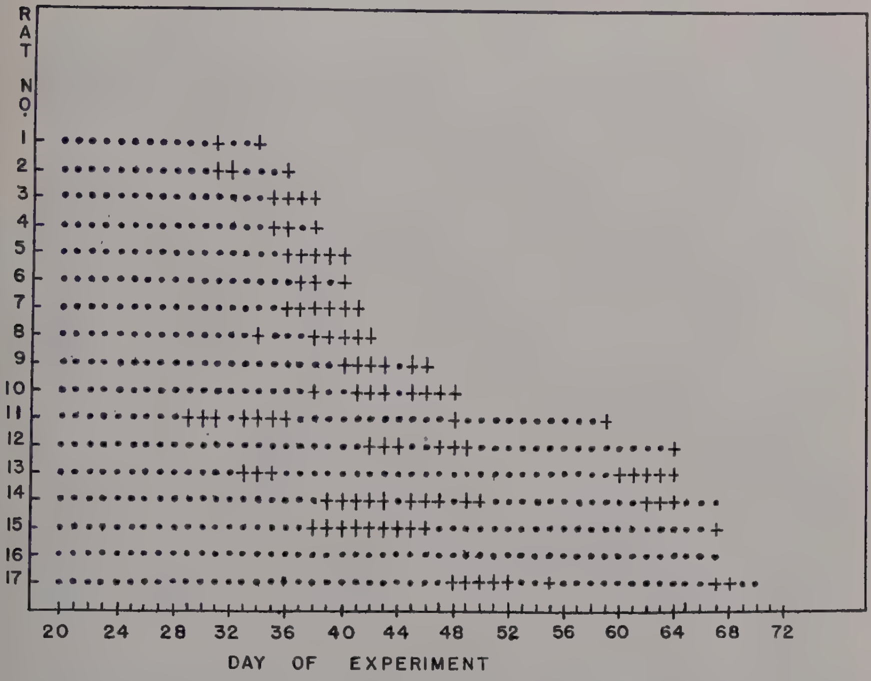

FIGURE 1. Daily urine bilirubin tests in 17 rats exhibiting, at autopsy, postnecrotic scarring of the liver (See TABLE 3). Testing began after the rats had been maintained on the necrogenic diet for 20 days and was continued until each animal died of a massive hemorrhagic necrosis of the liver.

of death in all animals) plus evidences of previous necrosis. As might be expected, all stages of healing as well as acute degeneration were seen. Sections through areas of the liver that appeared as depressions on gross inspection exhibited approximation of the larger vessels and bile ducts as a result of the disappearance of parenchymal cells. Fibrosis was inconspicuous. It was not possible to detect evidence of nodular regeneration. Despite careful searches we were unable to find in these livers that type of concentric nodule of hyperplasia so characteristically seen in rats with cirrhosis due to severe fatty infiltration. After a prolonged search of many sections, some areas of liver tissue that superficially resembled the pseudolobular pattern depicted by Abel *et al.*[5] (see their figure 14) were found. These areas also bore a resemblance to the illustration presented by Himsworth[4] (see his figure 27A, page 53). However, we were unable to demonstrate fibrotic bands extending between the small round areas of parenchymal cells in our sections. These small round areas of residual parenchymal liver cells often exhibited the early changes of necrosis, karyolysis, and karyorrhexis. We interpreted the areas in our material as "islands" of residual liver cells. They presented a pseudolobular pattern because there was a bandlike area of more advanced necrosis about them. They were regarded as areas of tissue that were being decreased in size rather than expanding in the fashion that seems to characterize the concentric nodules of regeneration in fatty cirrhosis (FIGURES 3 and 4).

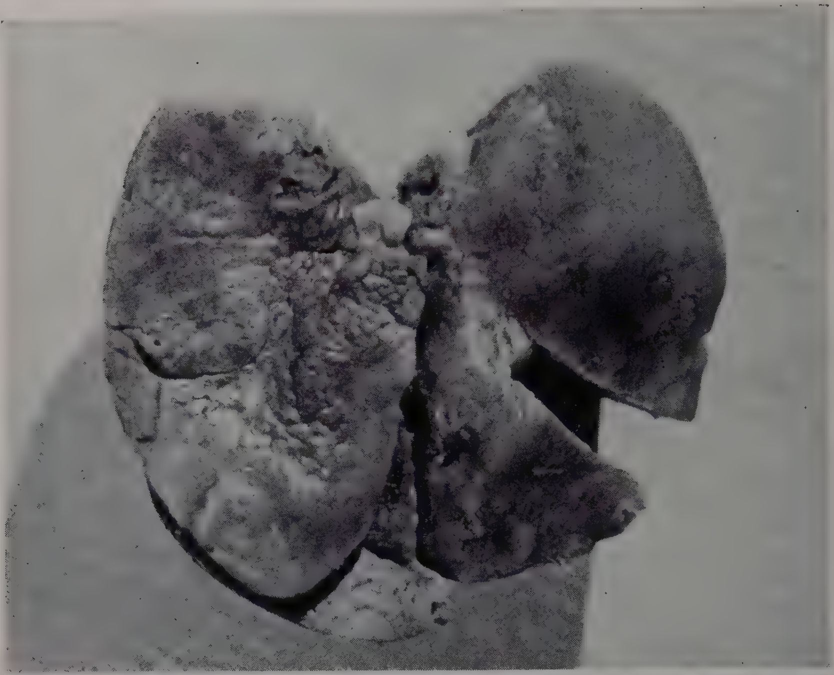

FIGURE 2. Massive hemorrhagic necrosis in a liver exhibiting postnecrotic scarring as the result of recurrent attacks of dietary necrosis. The specimen is from rat number 17 shown in FIGURE 1. The accessory lobes have been removed; the inferior surfaces of the three major lobes are shown. A block of tissue has been removed from the right lateral lobe.

The failure to produce an experimental form of postnecrotic cirrhosis in these animals might be attributed to their relatively short span of existence. The longest survival period was 70 days (FIGURE 1). It could be argued that, in the natural course of the disease, recurrent attacks of necrosis prevent the degree of regeneration and repair necessary to permit the development of cirrhosis. A means was sought that would stop the process of degeneration, or at least slow it down, once an initial attack had occurred. It is well established from the studies of Schwarz,[8] György,[9] and others[10] that the addition of tocopherol to the diet exerts a powerful protective effect against the development of dietary liver necrosis. The effect of the addition of tocopherol to the diet *after* the occurrence of an episode of necrosis was investigated. It was assumed that no further episodes of necrosis would occur following tocopherol administration. The assumption proved to be correct. The occurrence of an episode of necrosis was detected by the presence of a positive urine bilirubin test. The details of the experiment are presented in TABLE 4. A group of 31 male rats (initial weight 50 ± 5 gm.) was started on the necrogenic BY18 diet. Urine tests were performed daily after 20 days had elapsed. When the presence of an episode of dietary necrosis was detected, 12.5 mgm. of a tocopherol solution was injected intramuscularly and the diet was fortified by 1 mgm. of tocopherol in oil added to the daily ration. Fourteen animals failed to exhibit bilirubin in the urine. They were not given tocopherol. All died of massive

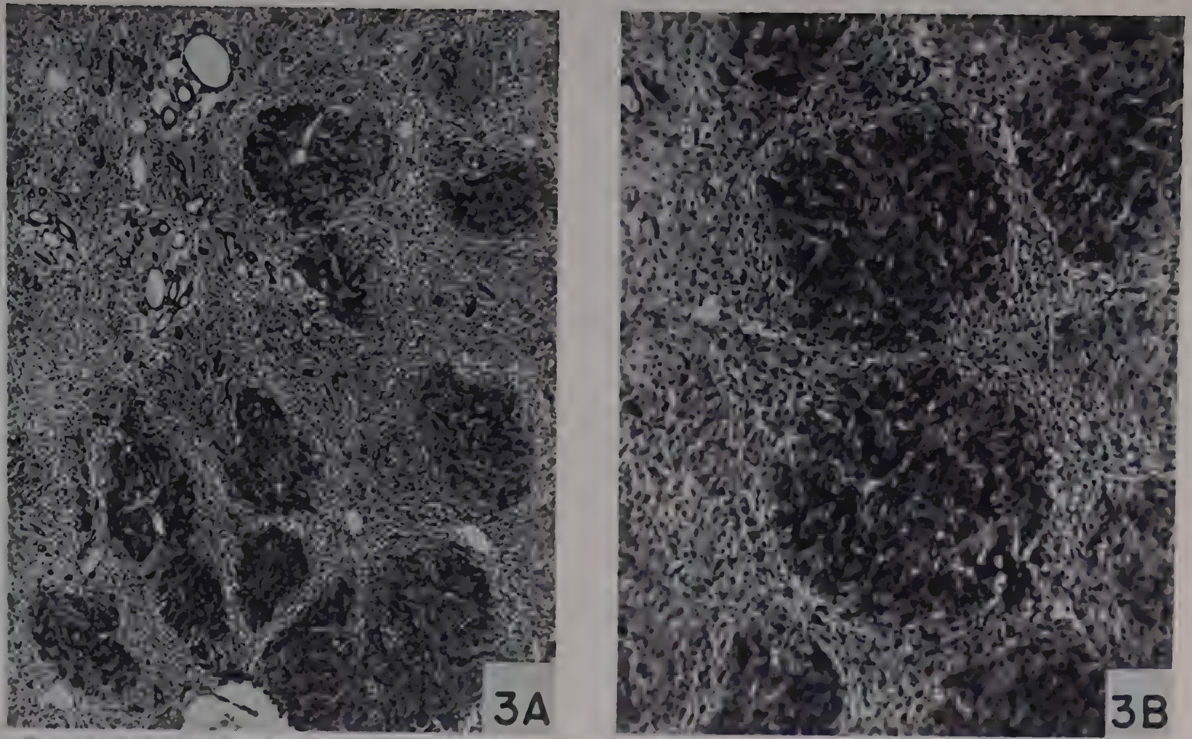

Figure 3. Section from liver of rat dead of massive hemorrhagic necrosis. Postnecrotic scarring of liver was apparent on gross examination. The round- or oval-shaped areas of stainable tissue represent residual parenchymal cells exhibiting an early stage of necrosis. These "islands" of residual cells are surrounded by the remnants of parenchymal tissue that have undergone necrosis. 3A—photomicrograph, 5 micra thick section, hematoxylin and eosin, ×50. 3B—same section, ×100.

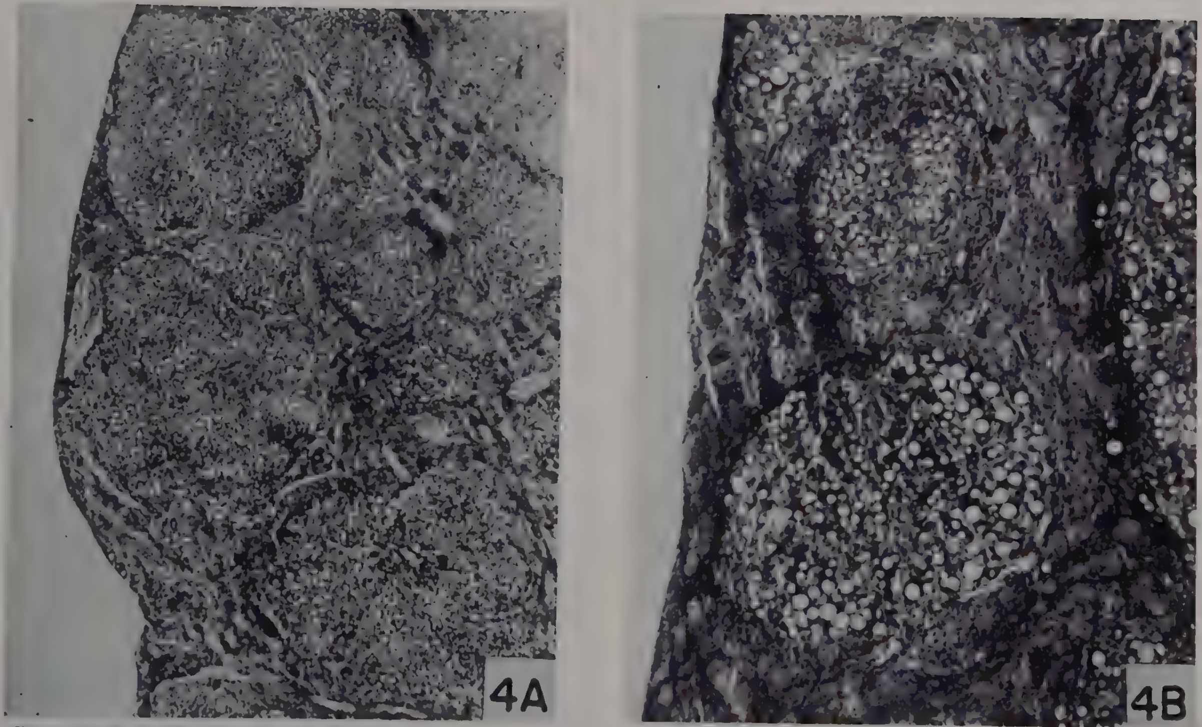

FIGURE 4. Section from liver of rat with fatty cirrhosis; animal maintained on choline-deficient diet 365 days. On gross inspection the regenerative nodules were clearly detectable as projections from the surface. On microscopic examination, such nodules exhibit compression of the surrounding fibrotic areas (condensed reticulin containing masses of ceroid). 4A. Photomicrograph, 5 micra thick section, azocarmine, ×50. 4B. Same section ×100.

TABLE 4

ADDITION OF TOCOPHEROL TO THE BY18 DIET AFTER AN INITIAL EPISODE OF HEPATIC NECROSIS

Rat no.*	First day U.B.† test positive	Day tocopherol started	Survival period (days)	Pathology	
				Massive necrosis	Postnecrotic scarring
1–14	—	—	28–58 (died)	14/14	6/14
15	33	35	35 (died)	+	+
16	36	37	37 (died)	+	+
17	42	42	43 (died)	+	+
18	40	40	41 (died)	+	+
19–31	30–46	30–46	163 (sacrificed)	0/13	12/13

* Male rats, initial weight 50 ± 5 gm.

† U.B.—Urine bilirubin.

liver necrosis. Four animals that were given tocopherol died within 48 hours. Apparently the pathologic process was so extensive at the time the attack was recognized that the disorder could not be stopped. Thirteen animals did survive. They were maintained on the BY18 diet fortified with tocopherol for an additional 117 to 130 days and were sacrificed as indicated in TABLE 4. Following the administration of tocopherol, the animals appeared to remain healthy. Billirubinuria usually disappeared after a few days. The subsequent daily tests revealed values recorded as "trace" from time to time but strongly positive reactions were not encountered. Daily testing was stopped after the 140th day.

When the animals protected by tocopherol were sacrificed, depressed scars of the liver were present in 12 of the 13 animals. This was most prominent on the inferior surface of the left lateral lobe. An example of this is shown in FIGURE 5. In this liver, the left lateral lobe is smaller than normal for a portion of the liver parenchyma has disappeared as a result of the earlier attack of necrosis. Veins that were once within the substance of the liver now may be seen to occupy a superficial position due to this loss of tissue substance. Sections through the involved areas revealed approximation of vessels and the larger bile ducts (FIGURE 6) and disappearance of parenchyma without evidence of extensive fibrosis. Nodular hyperplasia was not present. Sections of liver tissue from uninvolved areas appeared normal.

The results of this experiment suggested that recovery following an attack of nonfatal or submassive necrosis was followed by a disappearance of the involved portions of hepatic parenchyma including much of the supporting reticulin framework. The regenerative aspect was limited to a hyperplasia of the unaffected areas of the liver, a phenomenon best judged by the gross appearance of the uninvolved lobes of the liver. These rats exhibited changes similar to that seen in the residual lobes of the rat liver following partial hepatectomy; *i.e.*, thickening, appearance of rounded edges, and increased prominence of surface markings.

Addition of cystine. The protective effects of cystine in dietary liver necrosis

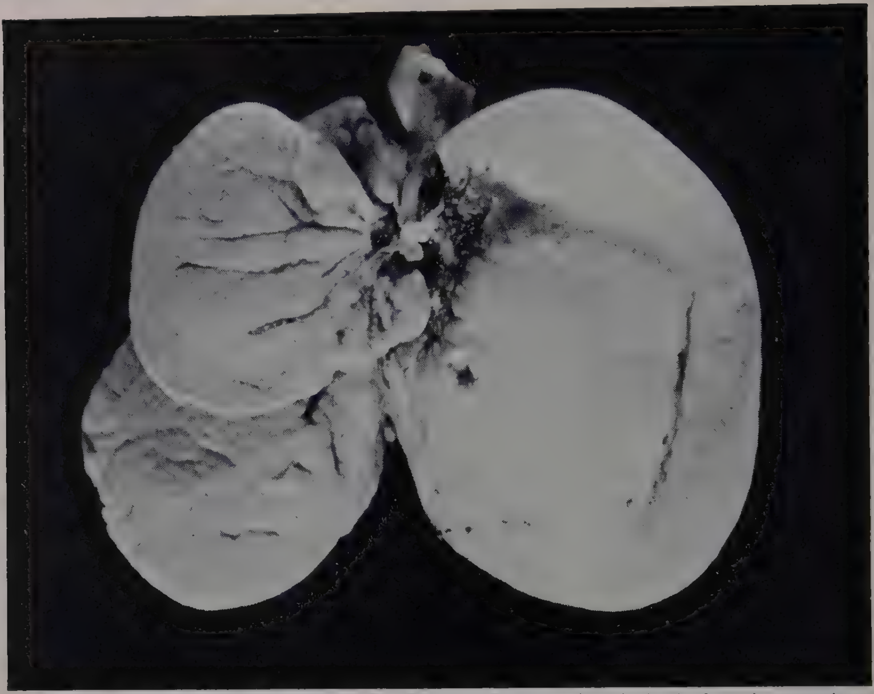

FIGURE 5. Late stage of postnecrotic scarring of the liver in a rat. An episode of submassive necrosis occurred 37 days after the start of the necrogenic diet. Tocopherol, administered then and subsequently, prevented further attacks. The animal was sacrificed 126 days after the original attack. The inferior surfaces of the three major lobes are shown. Note the diminution in size of the left lateral lobe as a result of the episode of necrosis. The superficial position of some of the larger veins in this lobe is apparent. An increase in the size of the right lateral and median lobes has taken place during the survival period.

are well established. The effect of the later addition of cystine to a necrogenic diet has been investigated by Schwarz.[11] In his studies, the supplement was added after the 26th day of the experiment. In the present study, cystine was added to the diet after a recognizable episode of dietary necrosis had developed. The amount given, 20 mgm. added to the 8 gm. daily ration allowance, was very small. This was deliberate. Complete protection was not desired. We hoped to favor the occurrence of repeated episodes of nonfatal necrosis. As it turned out, repeated attacks did not take place. The results of the study are shown in TABLE 5.

In this experiment, 12 animals failed to exhibit bilirubin in the urine or were obviously moribund when the test first became positive. It was possible to treat 13 rats and to study the effect of the late addition of the suboptimal amount of cystine. Five of the animals died of acute massive necrosis 4 to 96 days after the supplemented diet was begun. Bilirubinuria failed to develop and the onset of fatal dietary necrosis was not predicted. The remaining 8 animals were sacrificed on the 162nd day. Three animals showed gross and microscopic evidence of an early hemorrhagic necrosis at that time. This was probably coincidental. Had the time of sacrifice been postponed a few days, the three rats would probably have died of an extensive necrosis of the liver.

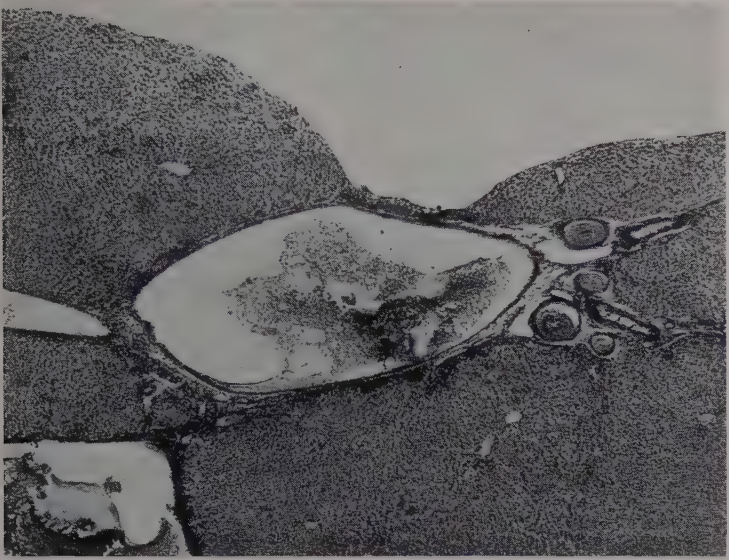

FIGURE 6. Section through area of healed postnecrotic scar in left lateral lobe of liver (See FIGURE 5). Following the early episode of submassive necrosis, further attacks were prevented by the addition of tocopherol to the necrogenic diet. Note disappearance of parenchymal tissue as evidenced by the close approximation of liver capsule to the large vein. Fibrosis is inconspicuous and nodular regeneration is absent. Photomicrograph, azocarmine stain, 5 micra thick section, ×50.

TABLE 5

ADDITION OF CYSTINE TO THE BY18 DIET AFTER AN INITIAL EPISODE OF HEPATIC NECROSIS

Rat no.*	First day U.B.† test positive	Day cystine started	Survival period (days)	Pathology	
				Massive necrosis	Postnecrotic scarring
1–12	—	—	42–60 (died)	12/12	5/12
13	51	52	56 (died)	+	+
14	40	40	98 (died)	+	+
15	52	52	134 (died)	+	+
16	50	50	139 (died)	+	−
17	58	58	154 (died)	+	+
18–25	25–58	25–58	162 (sacrificed)	3/8	7/8

* Males, initial weight 50 ± 5 gm.
† U.B.—Urine bilirubin.

Postnecrotic scarring of the liver was present in 11 of the 13 rats that had received the cystine supplemented diet (TABLE 5). Five also showed acute massive necrosis in addition. The degree of scarring as judged by the gross appearance was not very extensive. In three, the scarring could be classified as marked and in 8 as minimal. Histologic study of the livers of the animals sacrificed on the 162nd day revealed healed postnecrotic scars in all but one rat. These were similar to those seen in the tocopherol supplemented animals. In three animals, old lesions as well as evidence of the developing hemorrhagic necrosis described above was apparent. The liver of one animal appeared to be

TABLE 6

ADDITION OF AUREOMYCIN TO THE BY18 DIET AFTER AN INITIAL EPISODE OF NECROSIS

Number of rats*	First day U.B.† test positive	Day aureomycin started	Survival period (days)		Hepatic pathology			
						Degree of scarring		
			Range	Average	Acute necrosis	Absent	Minimal	Marked
7	—	—	34–69	49	7	3	3	1
21	32–47	—	32–70	45	21	2	9	10
19	32–45	32–45	38–102	68	19	2	6	11

* Males, initial weight 50 ± 5 gm.
† Urine bilirubin.

entirely normal. No evidence of nodular regeneration was encountered in any of the livers in this group of rats.

The failure to produce a form of postnecrotic cirrhosis in the above cited studies prompted an attempt to alter the natural course of the disorder by still another means. György *et al.*[12] had demonstrated the protective effects of various antibiotics in dietary necrosis of the liver. Aureomycin has a very definite protective effect when added to a known necrogenic diet at the start of the experiment. This agent was therefore employed in a fashion similar to that described for cystine and tocopherol in the previous studies. The results of the experiment are presented in TABLE 6.

As in the previous experiments, daily urine tests were started on the 20th day and continued until death occurred. As bilirubinuria was detected, alternate rats were given aureomycin.* Initially, the treated rat was given 1 ml. of a solution containing 20 mgm. of crystalline aureomycin hydrochloride via stomach tube. The drug, buffered with sodium glycinate, was the type prepared by the manufacturer for intravenous administration. The daily 8 gm. ration of the BY18 diet was thereafter supplemented by the addition of 20 mgm. of aureomycin hydrochloride (oral preparation). Food consumption was not measured, so that the actual amount of aureomycin consumed cannot be stated. Treated rats did gain some weight. Sixteen rats that survived had an average weight gain of 6.2 gm. during the first 28 days after aureomycin treatment was started. As can be seen from TABLE 6, treated rats survived longer than the controls. All eventually died of massive hemorrhagic necrosis of the liver. The results of the daily bilirubin tests for the treated animals are shown in FIGURE 7.

The incidence of postnecrotic scarring of the liver was the same in both the treated and the control rats, but the gross distortion of the liver in some of the animals that received aureomycin was more pronounced. The most severe example is shown in FIGURE 8. The lesion is most certainly an example of postnecrotic scarring. Hyperplasia of areas of unaffected parenchyma has undoubtedly occurred. Because of the restricting effect of the adjacent areas of older necrosis, the postnecrotic scarring, such regeneration will give a "nodu-

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* Supplied by Lederle Laboratories Division, American Cyanamid Corporation.

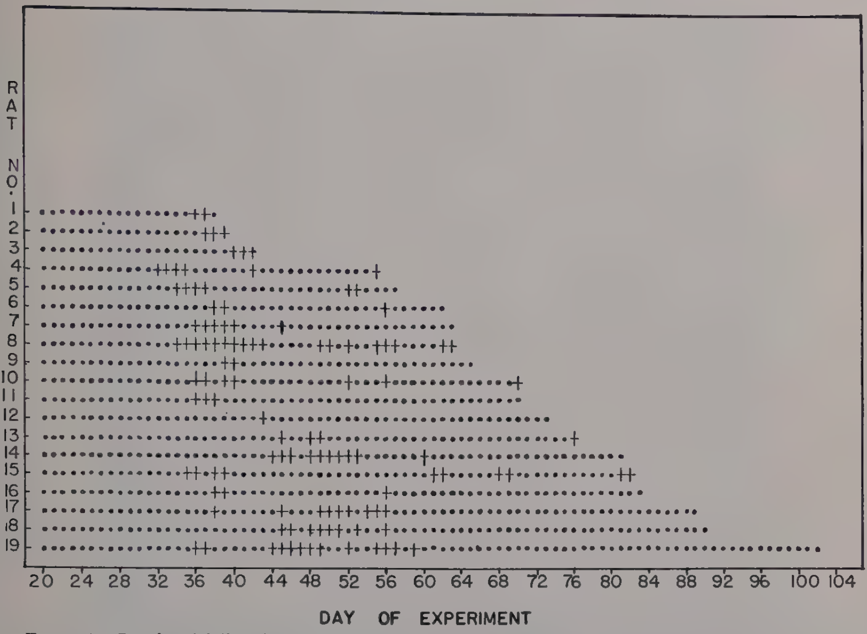

FIGURE 7. Results of daily urine bilirubin tests in 19 rats treated with aureomycin (See TABLE 6). The drug was administered on the first day a positive test was observed and continued until death of the animal. All rats died of massive hemorrhagic liver necrosis.

lar" appearance to the liver. This type of "nodule" is very clearly described in postnecrotic scarring following recurrent dietary necrosis by Himsworth.[4] In sections from the livers of these rats, we have not found convincing evidence of the presence of the type of nodule composed of a mass of parenchymal cells devoid of a lobular structure; *i.e.*, the concentric hyperplastic nodule. The type of fibrosis that could be found in some sections from these postnecrotic scarred livers is shown in FIGURE 9. This represents as striking a degree of fibrosis as we encountered and cannot be regarded as necessarily typical or characteristic of the entire group.

We have not succeeded in estimating the portal vein pressure in animals exhibiting postnecrotic scarring of the liver. Daniel, Prichard, and Reynell[13, 14] have done this in rats with fatty cirrhosis and cirrhosis due to repeated carbon tetrachloride exposures. It would be very worth while to study the portal blood flow in postnecrotic scarred livers by the radiographic techniques described by the above investigators. We have not been able to do this, but have employed a crude method of studying the effect of postnecrotic scarring on the circulatory system within the liver. This has consisted of the preparation of plastic (vinyl acetate) casts of the portal and hepatic venous system. Casts prepared from liver lobes that have been affected by postnecrotic scarring show a pattern quite different from that of the normal or that of a rat with an advanced stage of fatty cirrhosis. In postnecrotic scarring, the larger veins lie close together and the entire picture is one that would be expected where there is disappear-

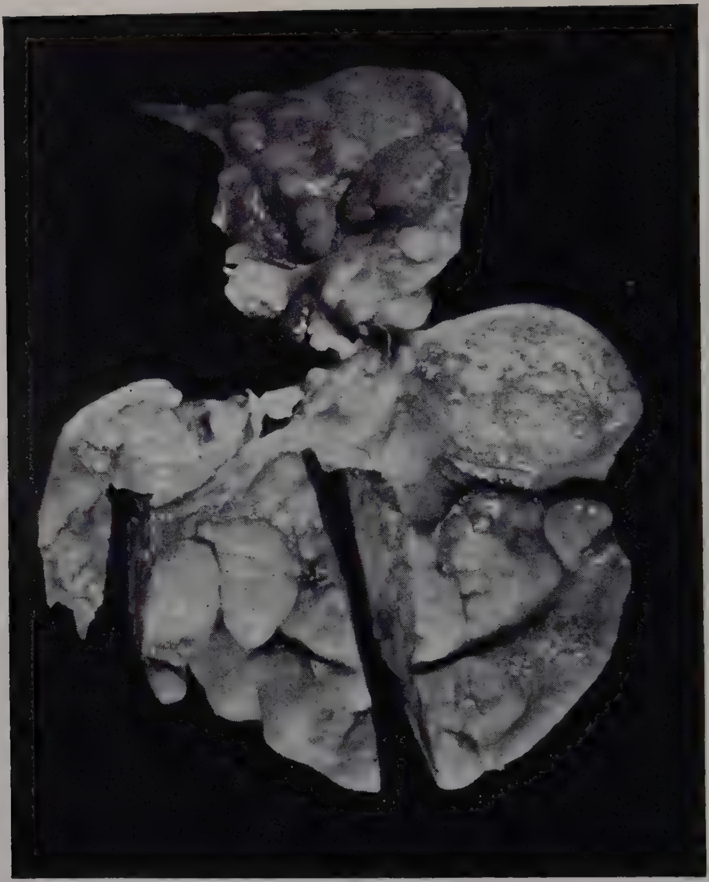

FIGURE 8. Postnecrotic scarring of the liver (specimen from rat number 8 in FIGURE 7). The inferior surfaces of the right lateral, the median and the accessory lobes are shown. The latter has been reflected upward. The left lateral lobe has been removed. The liver used for this illustration represents the most striking example of gross hepatic distortion encountered in these investigations.

ance of parenchyma. The loss of supporting framework permits collapse and approximation of the residual structures, *i.e.*, the larger veins. The resulting picture has been compared to an atelectasis of a lobe of the lung. When compared with a similar preparation of a normal liver lobe, the contrast is quite striking. Casts of veins from lobes of cirrhotic livers (fatty cirrhosis in the rat) show quite clearly the compressive effects of the expanding regenerative nodules. This feature of cirrhosis that has recently claimed the attention of Kelty, Baggenstoss, and Butt[15] and of Popper, Elias, and Petty[16] was not seen in the venous casts of postnecrotic scarred lobes that we prepared.

DISCUSSION

It is apparent from these observations that the employment of diets designed to produce recurrent bouts of liver necrosis does not offer a satisfactory means

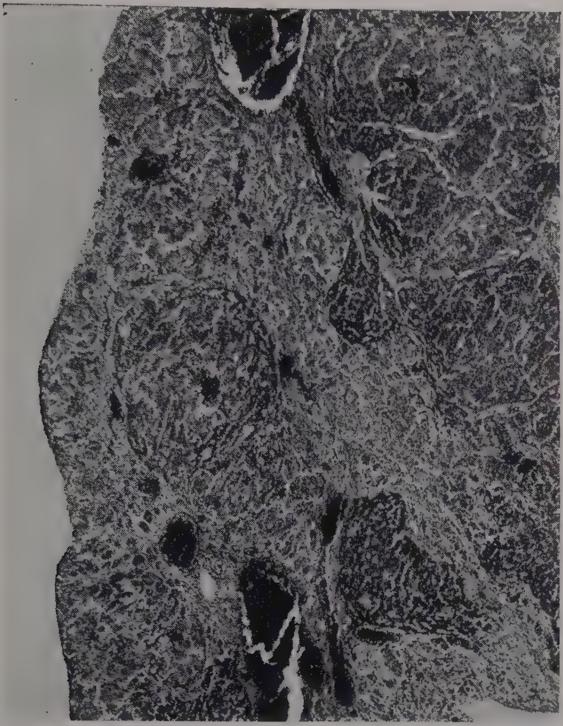

FIGURE 9. Section from liver shown in FIGURE 8. Acute hemorrhagic necrosis superimposed on older stages of necrosis. The latter is in various stages of resolution. The irregularity of the surface (left side of illustration) is produced by contraction at sites of earlier areas of necrosis. The round mass of tissue just to the left of the center in the photomicrograph has only a thin rim of reticulin fibers about it. The areas that appear gray in this illustration represent areas of necrosis rather than the broad bands of fibrosis one encounters in cirrhotic livers.

of inducing experimental cirrhosis. In the first place, this does not represent a very flexible or convenient experimental tool. The time of occurrence of necrosis is unpredictable. Bilirubinuria, when it does occur, almost certainly develops after necrosis has begun. Its recognition serves a useful purpose, but it cannot be regarded as a means of detecting impending or threatened necrosis. Actually, the investigator has relatively little control of the situation as far as determining the frequency or the severity of the episodes of necrosis. This then is quite different from the experimental procedure in which one employs repeated exposures to a toxic agent such as carbon tetrachloride. In that case the severity of the recurrent episodes of liver injury can be partially regulated by varying the dose and the frequency of administration of the insulting agent. However, recurrent dietary necrosis should not be regarded as just another experimental means of producing liver injury. The pathologic lesions that result are quite unique in many respects.

The natural course of dietary hepatic necrosis is quite variable and depends on the experimental conditions. As indicated for the present studies, young growing male rats developed fatal episodes of necrosis four to ten weeks after they were placed on the diet. The lack of uniformity in the time of development of the hepatic lesions appears to be a characteristic feature of the disorder. No satisfactory explanation has been offered and the present studies shed no light on this interesting aspect. Nor does there appear to be any satisfactory explanation as to why necrosis is massive and fatal in the first attack in some animals and submassive in others. The events that may occur during the course of the disorder, including the development of intermittent bilirubinuria, have been clearly described by Himsworth.[4] Our experience has been in accord with this description. We have not witnessed the development of ascites or pleural effusion in our animals, but this may be the result of a difference in experimental conditions. None of the rats (FIGURE 1) survived the unaltered diet for longer than 70 days for their lives were ended by an episode of massive necrosis.

The interpretation of the pathologic lesions that resulted from recurrent dietary necrosis was difficult. Except where tocopherol or cystine was given, all of the animals died of massive hemorrhagic liver necrosis. Consequently, this lesion was superimposed on the older lesions that were in various stages of resolution. The attempts to modify the natural course of the disease by adding cystine or aureomycin to the diet actually did not help to clarify this situation very much. All of the animals that received aureomycin and 8 of 13 animals that received cystine also died of a massive hemorrhagic necrosis. These agents were added in a deliberate attempt to permit the repeated occurrence of more episodes of dietary necrosis than would take place in the natural course of events. In the case of aureomycin there is some evidence that such did occur (compare FIGURES 1 and 7). Cystine, when added in suboptimal amounts, apparently failed to accomplish this goal. The animals lived for variable periods but did not have recurrent episodes of necrosis. The interpretation of the hepatic pathology still presented the same problem; namely, that of a massive necrosis superimposed on the older lesions.

The problem of interpretation of the hepatic pathology was simpler in the rats that received tocopherol and in five of the rats that received cystine. In these animals, protection from further episodes of necrosis appeared complete. With the exception of two animals where no evidence of previous necrosis could be detected, the others exhibited scars. There appeared to be a complete disappearance of the parenchymal structures in such areas. The absence of any significant amounts of residual fibrosis seemed to indicate that, in the process of necrosis, the reticulin framework had undergone dissolution and had been removed along with the disintegrated hepatic cells. The regeneration of the liver that followed this single episode of necrosis appeared to be similar to that which follows a partial hepatectomy; *i.e.*, enlargement of the residual and unaffected lobes.

As stated, these investigations were undertaken in an attempt to produce an experimental form of postnecrotic cirrhosis. The question of whether a given hepatic lesion is or is not cirrhosis is sometimes difficult to answer. The rat subjected to prolonged fatty infiltration of the liver as a result of maintenance on a choline-deficient diet regularly develops a lesion that is acceptable as an example of cirrhosis. Two prominent and conspicuous features are fibrosis and concentric nodules of regeneration. Neither of these features were conspicuous or frequent in the livers of rats that had undergone recurrent episodes of dietary necrosis in our experiments. The type of nodule composed of a mass of parenchymal cells devoid of lobular structure, the concentric hyperplastic nodule, was not identified in sections from the livers of our animals with any degree of certainty. Admittedly, we do not have accurate criteria for the recognition of such structures. In fatty cirrhosis in the rat, they appear to be readily recognizable and their compressive effects on adjacent veins are usually readily discernable in microscopic sections.

It is not felt that the hepatic lesions we have observed in the rats described in these investigations warrant the designation, postnecrotic cirrhosis. It is of some interest in the problem of the pathogenesis of cirrhosis that recurrent episodes of dietary necrosis do not readily, at least, result in lesions characterized by fibrosis and nodular regeneration.

Summary

(1) Recurrent episodes of dietary hepatic necrosis occurred as a spontaneous feature in young growing male rats maintained on a yeast diet. Grossly discernable scarring of the liver involving two or more lobes occurred in 25 per cent of a group of 70 animals.

(2) As a means of following the course of the disease, daily urine tests for the presence of bilirubin were performed after each rat had been maintained on a necrogenic diet for 20 days. The occurrence of some episodes of nonfatal dietary hepatic necrosis could be detected by the presence of bilirubinuria.

(3) In one experiment, an adequate amount of tocopherol, a known protective substance, was added to the diet after the occurrence of a recognizable episode of necrosis. This permitted prolonged survival. In 12 of the 13 animals in the experiment, previous sites of hepatic necrosis were represented by healed scars. Such areas exhibited approximation of the residual larger vessels (veins and bile ducts) with nearly complete disappearance of all remnants of previous parenchymal structures and a minimum of residual fibrosis.

(4) In another experiment, an inadequate amount of cystine, also known to be a protective substance, was added to the diet after an episode of necrosis. This prolonged life but did not prevent eventual fatal massive necrosis in all of the animals. Recurrent episodes of necrosis were not manifest during the period of supplementation. The resultant pathology was similar to that described above (3) plus evidences of a recent massive necrosis that developed in 8 of the 13 animals in the experiment.

(5) Aureomycin was administered to alternate rats after each had undergone an episode of necrosis during the course of an experiment. The 19 treated animals survived longer (average 68 days) than did 21 untreated controls (average 45 days). All animals died of a massive liver necrosis. The incidence of postnecrotic scarring was equal in the two groups. The severity of the gross distortion of the liver was slightly greater in the treated group. All stages of recent and previous necrosis were present in the livers of both groups.

(6) Two criteria have been demanded for the acceptance of an experimental lesion as an example of cirrhosis: extensive fibrosis and conspicuous concentric nodules of hyperplasia. Both features are readily apparent in the rat with

cirrhosis due to prolonged fatty infiltration. Neither of these features were prominent in the rats maintained on the necrogenic yeast diet. The production of an acceptable form of experimental postnecrotic cirrhosis was not accomplished in these experiments.

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Discussion of the Paper

Doctor Hans Popper: The well-illustrated presentation of Doctor Hoffbauer deals with an extremely important aspect; namely, the clarification of the histogenesis of postnecrotic cirrhosis by experimental methods. He is to be congratulated on a well-carried-out study. The use of bilirubinuria as an indication of hepato-cellular degeneration is a simple but convincing device. In human pathology there are two, and possibly three, types of lesions which deserve the term "postnecrotic cirrhosis." Laennec's cirrhosis, including the fatty type, may also develop as a result of necrosis; however, this necrosis involves only very small parts of the lobule. In contrast, the postnecrotic cirrhosis is the result of massive necrosis, involving whole lobules with resulting collapse of their framework. In the cirrhotic stage this is characterized by wide bands of connective tissue in which the original portal and central fields have been approximated. This lesion can only involve a part of the liver if survival should be possible. In the autopsy material of Cook County Hospital, this is most commonly seen as secondary collapse as a result of massive necrosis occurring in a Laennec's type of nutritional cirrhosis. This lesion hardly deserves the name "postnecrotic cirrhosis." The second common form is the cirrhosis with the story of a chronic progressive deterioration usually associated with jaundice and often preceded by what could be a viral hepatitis. As Doctor Hoffbauer points out, this does not respond to nutritional therapy. At autopsy, characteristic large bands resulting from collapse are noted; the surrounding parenchyma is not normal but disorganized although not frankly cirrhotic. This type of postnecrotic cirrhosis can be explained by a combination of massive necrotizing insults to some parts of the liver with multiple focal necrotizing injuries in the rest of the liver, all due apparently to the same cause. Only rarely is the third picture seen in which large bands traverse the liver in which the remaining parenchyma has normal architecture and normal liver cells; no functional liver damage or jaundice is observed. This is the coarse nodular cirrhosis of Marchand, a monument to a previous insult. This last type of lesion is similar in histogenesis to the experimental lesion of Doctor Hoffbauer. It fails to show portal hypertension, lack of compression of hepatic veins and the characteristic regenerative nodules with plates several cells in thickness. This condition probably results from one major localized insult. usually not repeated nor protracted, and probably sparing of the surrounding tissue. In the collapsed lobules no liver cells are left for regeneration. However, the lesion of Doctor Hoffbauer differs from this last type of postnecrotic cirrhosis in that apparently no broad bands of connective tissue develop. Doctor Elias and I have demonstrated that collapse following massive necrosis, at least in man, involves not only the necrotic area but produces breaks due to stress in the surrounding parenchyma. In these cracks connective tissue septa develop dissecting the noncollapsed tissue. No evidence for this process can be found in the animals of Doctor Hoffbauer. This may be explained by the known difference in the connective tissue stroma in rats and man, it being far denser in the latter. Nevertheless, the studies of Doctor Hoffbauer point out that one massive insult is not necessarily followed by cirrhosis formation and that, at least for the common form of postnecrotic cirrhosis in man, probably lingering injury to the entire parenchyma is required in addition to the massive localized insult. These studies therefore fill a gap in our knowledge.

THE EFFECT OF ENVIRONMENT AND MODE OF FEEDING AND OF REARING ON THE PRODUCTION OF ACUTE DIETARY LIVER NECROSIS IN THE RAT

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My aim in the experiments described below was to define the conditions under which young male rats would become susceptible to acute dietary liver necrosis.

In the first series of five experiments,[1, 2] two conditions were examined simultaneously; (1) the influence of environment; and (2) the mode of feeding, *i.e.*, whether the rats were allowed to eat to appetite or not. In the second series of three experiments,[3] a method was found of adjusting the susceptibility of groups of rats to the development of acute liver necrosis. With such a method, more detailed comparisons can be made between rats likely to develop liver necrosis in a short time and those which would take longer to do so, or would not develop the disease at all.

The Interactions between Environmental Temperature and Mode of Feeding

Environments. The environments are described in detail in previous papers.[1, 2] It should be emphasized that, although these experiments were designed in the first instance to study the environment, only the effect of the dry-bulb air tempeature has been examined in detail. Briefly the usual ranges of environmental temperature were 35–45°F.; 40–50°F.; 60–64°F.; 63–70°F.; 70–74°F.; 72–76°F.; 70–78°F.; and 88–92°F.

Diets. The following three diets were used: (1) During pregnancy and the first 17 or 19 days of lactation, the Rowett Research Institute stock-rat-cube[4] together with cow's milk was given. This diet was followed by (2) a semi-synthetic preliminary high-protein diet containing casein 16 or 19 ("vitamin-free" Glaxo Laboratories Ltd., Middlesex, England), brewers' yeast 3 (Pharmaco-Chemical Products, Ltd., London), sucrose 68 or 65, commercial lard 7, McCollum's salts 4, cod liver oil (B.P.) 2 per cent, and synthetic B vitamins but without added Vitamin E. These diets were available to the young. (3) From the sixth week of age, the young were fed a low-protein diet containing, as the source of protein, 8 per cent casein and 3 per cent yeast, the sucrose being increased to 76 per cent. The yeast was added to supply the extra vitamins which may be required by animals exposed to cold. The reasons for these procedures are explained in a previous paper.[1]

Management of the experiments. The rats were weaned on the 24th day of age. When the low-protein diet was offered, one group of rats in each environment was allowed to eat to appetite and, except in experiment 3, one or two groups in each environment were restricted in different degrees in their food intake.

Results

The results of the first experiment are shown in TABLE 1. It is evident that in an environmental temperature of 63–70°F. the rats which ate to appetite

TABLE 1
EXPERIMENT 1
INCIDENCE OF DEATHS IN RATS GIVEN EXPERIMENTAL DIET*

Group	Management		No. tested	No. dead with acute liver necrosis	Median survival time (days)	No. dead without acute liver necrosis
	Housing; Usual range of temperature °F.	Feeding				
A	Animal house (63–70°) (heated)	"Ad lib."	8	7	19	0
B		Restricted	9	1†	—	0
D	Rabbit hutch (40–50°) (unheated)	"Ad lib."	8	0	—	0
E		Restricted	9	0	—	4

* Survivors killed 53 days after feeding low-protein diet.
† Rat died 53 days after feeding low-protein diet.

died of acute liver necrosis while those with a restricted food allowance or those in the lower environmental temperature (40–50°F.) did not develop the disease. When the food allowance was restricted in the lower environmental temperature, four among nine rats died without liver necrosis.

The second and third experiments were conducted in the winter of 1949–1950 and in the spring of 1950 with better temperature control of the heated environments. In experiment 2 the usual range of the air temperature of the unheated environment was 40–50°F., that of the heated environment 70–74°F.; *i.e.*, several degrees Fahrenheit above the temperature studied in experiment 1. In experiment 3, groups of rats in two heated environments, 70–74°F. and 60–65°F., and two unheated environments were studied. In this experiment, only one "restricted" group of rats was studied (group 3E TABLE 2). These rats were put into the environment of 60–65°F., so that the conditions at the beginning of experiment 1 were simulated. The results (TABLE 2) of experiment 3 confirmed those of experiment 1, while those of experiment 2 differed in two main respects: (1) in the heated environment of 70–74°F., the incidence of acute liver necrosis was the same among the rats "restricted" in their food allowance

TABLE 2
EXPERIMENTS 2 AND 3
INCIDENCE OF DEATHS IN RATS GIVEN EXPERIMENTAL DIET

Experiment	Group	Management		No. of rats tested	No. dead with acute liver necrosis	Median survival time (days)	No. dead without liver necrosis
		Housing; Usual range of temperature °F.	Feeding				
2	A	Animal house (70–74)	"Ad lib."	9	8	43	0
	B		Restricted	9	8	40	0
	E	Unheated wooden shed (40–50)	"Ad lib."	10	3	—	0
	F		Restricted	10	1	—	3
3	A	Animal house (70–74)	"Ad lib."	12	11	19	0
	B	Heated wooden shed (60–65)	"Ad lib."	12	8	26	0
	D	Rabbit hutch (35–45)	"Ad lib."	12	0	—	1†
	E	Heated wooden shed (60–65)	Restricted	8	0	—	0

† Killed, because of severe otitis media, on 21st day after being first given the experimental diet.

TABLE 3

EXPERIMENTS 1, 2, AND 3

FOOD EATEN BY "AD LIB." GROUPS* AND DEGREE OF FOOD RESTRICTION

Expt. no.	Envirn. temp. °F. Usual range (extreme range)	Average food eaten, g./rat/day by "ad lib." group ( )	Average food offered, g./rat/day to restricted group ( )	Average degree of food restriction "ad lib." / restricted	Remarks
1	63–70	5.8 (A)	5.0 (B)	1.15; 1.09	i. Only 2 rats studied for 1 week in "ad lib." group A. ii. One rat out of 9 died with liver necrosis in restricted group B when environmental temp. reached 80°F.
	40–50	11.8 (D)	8.8 (E)	1.3	Four rats died *without* liver necrosis in restricted group E. Degree of restriction greater than 1.3 at that time.
2	70–74	4–5→3.9 (A)	3.6 (B)	1.2→1.1	Incidence of liver necrosis the same in groups A and B; *i.e.*, 8 out of 9.
	40–50	10.8 (E)	7→9.4 (F)	1.2	Three rats died *without* liver necrosis in group F when degree of restriction was 1.4; 1.34 and 1.35. Temps. 38, 28, and 40°F.
3	60–65	7.0 (B)	4→6 (E)	1.6→1.4	No rat died in restricted group E.

* Only groups relevant to the argument are shown. For details of other groups see Naftalin[1].
→ = increased or reduced as experiment progressed.

(group 2B TABLE 2) as among those fed "ad lib." (group 2A); (2) the median survival times, after first giving the experimental low-protein diet, were 40 and 43 days, as compared with 17 and 19 days in experiments 1 and 3. The reason for this second difference is not fully apparent, though it may be due to seasonal changes.

In seeking to explain why the "restricted" rats in experiment 2 behaved differently from the "restricted" rats in experiments 1 and 3, the amounts of food eaten and the degree of food restriction were then examined. The results are shown in TABLE 3. In experiment 2, the rats fed "ad lib." (group 2A) did not eat as much as those in corresponding groups in experiments 1 or 3 (groups 1A and 3B). The average amount eaten by group 2A decreased from 4.5 to 3.9 gm./rat/day as the experiment progressed. It had been decided at the beginning of experiment 2 to offer 3.8 gm. of food daily to each rat in group 2B, the "restricted" group but, although after 8 days this was reduced to 3.6 gm., the ratio of food eaten by the group fed "ad lib." to the "restricted" group did not rise above 1.1. It seemed, therefore, that the degree of food restriction had not been great enough at 70–74°F. to prevent death from acute liver necrosis. This postulate was tested in experiment 4, when two degrees of food restriction were studied at two environmental temperatures, 60–64°F. and 70–78°F. The degrees of food restriction were such that the amounts of food

TABLE 4

RESULTS OF FOOD RESTRICTION AT VARIOUS ENVIRONMENTAL TEMPERATURES

(*Experiments* 1–5)

Environmental temperature °F. (usual range)	Expt. no.	Group average degree of food restriction	Results (restricted groups)
88–92	5	1.7	Death without liver necrosis.
		1.2	Protection against liver necrosis. Rats live.
70–78	4	1.6	Not complete protection against liver necrosis.
		1.3	No protection against liver necrosis.
70–74	2	1.2→1.1	No protection against liver necrosis.
60–64	4	1.6	Death *without* liver necrosis.
	3	1.6→1.4 (lowest 1.25)	Protection against liver necrosis. Rats live.
	4	1.2	No protection against liver necrosis.
About 63	1	1.15; 1.09*	Protection against liver necrosis.
Below about 45	1 & 2	When ratio was about 1.35	Death *without* liver necrosis.

* Only 2 rats studied for one week in the corresponding "Ad Lib." group.

eaten by groups allowed to eat to appetite divided by the amounts of food offered to the restricted groups in the same environmental temperature, gave ratios of 1.2 and 1.6 at 60–64°F. and 1.3 and 1.6 at 70–78°F. In experiment 4, at the higher environmental temperature of 70–78°F., the rats which were allowed to eat to appetite all died of acute liver necrosis (median survival time, 14 days). Liver necrosis was not prevented in three among 12 rats by a degree of food restriction of 1.6 at this temperature while, at the lower temperature of 60–64°F., this degree of food restriction resulted in the death of three among 12 rats without liver necrosis. Liver necrosis was not prevented when the degree of food restriction was 1.2 in the environmental temperature of 60–64°F. The degree of food restriction necessary to prevent death at 60–64°F. would seem to lie about 1.4—the ratio used in experiment 3 (TABLE 4).

Experiment 5 was an extension of these observations. Littermates were allocated to four groups: one group of rats, allowed to eat to appetite in an environment of 88–92°F. was compared with a similarly fed group housed at 74–76°F.; two groups with different degrees of food restriction, 1.2 and 1.7, also at 88–92°F., were compared with the group fed to appetite in this environment. From this experiment it was concluded that an environmental temperature of 88–92°F. was higher than the optimum temperature for the production of acute liver necrosis.[2] This conclusion was reached because, at this temperature, the group of rats allowed to eat to appetite survived longer than their littermates housed at 74–76°F., and because the low degree of food restriction of 1.2 prevented liver necrosis. The range 80–88°F. has not yet been studied, but an environmental temperature of about 75°F. is suitable for a high incidence of the disease. It was thus apparent that it was the degree of food restriction in relation to the environmental temperature that determined whether a rat survived or died either of liver necrosis or of inanition (TABLE 4). Although the trend is clear, it should not be inferred that these ratios are fixed values. Possibly other values may be obtained, if the rats are not reared in the same manner or if the composition of the diet is different from that used in these experiments. These relationships have been illustrated in a graph in a previous paper.[2]

Behavior of the rats in the different environments. There were marked differences in the activity of the rats in the heated and cold environments and between those rats allowed to eat to appetite and those whose food allowance was restricted. The rats in the heated environments of 70–78°F. or 88–92°F. which ate to appetite were sluggish and often drowsy. They tended to lie in a corner with nose down and back humped. In a slightly cooler environment of 60–65°F. the rats fed to appetite were, on the whole, more active and the most active survived. The rats fed to appetite in the unheated environments were still more active. In experiments 1, 3, and 5 conducted in the spring or early summer, the rats were more active than those in experiments 2 and 4 conducted in the winter.

The most active rats were those whose food allowance was adequately restricted. These rats ran and jumped about their cages with great alacrity. After the second or third week, all the food given was eaten within 15 minutes or sooner. When this happened, it could be predicted that a rat was unlikely to die of liver necrosis.

Discussion

Environmental temperature and food restriction. Since the same diet was fed to all the rats in experiments 1 to 5, the production of acute liver necrosis cannot be attributed solely to the composition of the diet.

Restricted food intake leads to undernutrition, and this reduces the metabolic rate. A low environmental temperature, and possibly also an environmental temperature above the zone of thermoneutrality, increases the metabolic rate. Since these conditions prevent liver necrosis, a paradox arises which could be explained by the following assumption. In the region of thermoneutrality, the liver is responsible for a large part of the metabolism. At a lower environmental temperature or in undernutrition, the other tissues play a larger part. It should be noted that the rats which were adequately restricted in their food intake so as to prevent liver necrosis became very active and hence the muscles were probably responsible for even a greater part of the metabolism than in those rats fed to appetite which lay quiescent in their cages. The effects of feeding may provide a stimulus so that the metabolism of the liver becomes suddenly increased. This increase would be most marked in rats fed to appetite in an environmental temperature near the zone of thermoneutrality for the rat; *i.e.*, 80–90°F. Anderson and Lusk[5] in their study of the metabolism of a dog which was alternately adequately fed and starved came to the conclusion that it was the (dietary) condition of the body and not a large influx of food on the previous day which determined the height of the basal metabolism; *i.e.*, the specific dynamic effect of food was diminished by previous starvation. Exposure to a cold environment can also lower the specific dynamic action of foodstuffs (Rübner, quoted by Brody[6]).

These arguments and speculations indicate that an essential problem is the study of the metabolism of rats under different feeding and environmental conditions. An attempt should be made to evaluate the metabolism due to the liver and that due to the rest of the body.

Age at Weaning, Survival Time and Incidence of Acute Liver Necrosis

In general, a high incidence of acute liver necrosis had been produced when the rats were suitably housed and fed. The next step was to find a method whereby the susceptibility of batches of rats to the development of acute liver necrosis could be adjusted. This objective was achieved by weaning littermates early on their 17th day of age or late on their 25th.[3]

TABLE 5 compares the results of experiment 6, environmental temperature 72–76°F., with the results of the groups in experiments 1 to 5 and one unpublished experiment in a similar environment. In experiments 7 and 8, littermates were compared. Groups of rats weaned early suffered a significantly higher incidence of liver necrosis and developed the disease in a shorter time than their littermates weaned late. In experiment 7, among a group of 13 rats weaned on the 17th day, twelve died of acute liver necrosis (median survival time, 10 days), as compared with ten deaths among 14 of their littermates weaned on their 25th day (median survival time, 28 days). This difference in median survival time is highly significant ($P < 0.001$). Both groups of rats were fed to appetite on the semisynthetic diets described on p. 862. A similar result was obtained in experiment 8 with 18 per cent brewers' yeast (Pharmaco-Chemical Products Ltd., London) as the source of protein in the experimental low-protein diet.[3] This method of rearing, thus obtaining groups of rats of high and low susceptibility to acute liver necrosis, is now being used to test the effect of substances which may have only slight inhibitory or acceleratory properties towards liver necrosis. Moreover, by investigating differences between littermates weaned early or late, another new approach is opened to the study of acute dietary liver necrosis. Statistical analyses have demonstrated a significant difference between litters even when a highly inbred strain such as the black and white hooded Lister rat of the Rowett Institute stock was used. This method of management (*i.e.*, by early and late weaning) gives a more reliable basis for comparison than selecting rats at random. Incidentally, this method of obtaining groups of rats of different susceptibility may prove useful in studying diseases other than acute liver necrosis.

TABLE 5

AGE AT WEANING, INCIDENCE OF ACUTE LIVER NECROSIS AND MEDIAN SURVIVAL TIME IN EXPERIMENT 6 AS COMPARED WITH 6 PREVIOUS RESULTS*

Experiment	Day of age when weaned	No. of rats tested	No. dead with acute liver necrosis	Median survival time†
6 (black and white rats)	17th	11	11	6
6 previous experiments* (albino rats)	24th	61	58	14, 15, 15, 17, 19, 43

* Experiments 1–5 and one unpublished experiment. All rats fed to appetite; environmental temperatures: experiment 1, 63–70°F.; experiments 2–5, 70–78°F.; experiment 6, 72–76°F.

† Days after eating experimental diet; *i.e.*, days after 38th day of age.

The reasons for the differences between the rats weaned early or late are under investigation. Since the preliminary high-protein diet fed to the mothers from the 17th day of lactation may in itself lead to necrosis in the young,[7] it would seem that the rats weaned later either (1) had a nonspecific improvement in their nutrition or (2) they received more of a protective substance which, (a) although present only in traces in the diet, could be concentrated in the milk or which (b) was synthesized in the mother's body and secreted in the milk.

Summary

(1) A high incidence of acute liver necrosis was produced in young male rats, when the animals were housed in a suitable environment and allowed to eat to appetite of a low-protein diet containing 8 per cent casein (Glaxo "vitamin-free"), 3 per cent brewers' yeast (Pharmaco-Chemical Products), 7 per cent lard, 2 per cent cod liver oil (B.P.), 76 per cent sucrose, and 4 per cent McCollum's salt and B vitamins, but without added vitamin E.

(2) Of the ranges of environmental temperature studied, the best temperature for the production of acute liver necrosis was 70°–78°F.

(3) At all environmental temperatures studied, the incidence of acute liver necrosis was reduced or prevented by a sufficient degree of food restriction, but too severe a degree of food restriction led to death without liver necrosis. Whether a rat survived or died either of necrosis or of inanition depended on the degree of food restriction in relation to the environmental temperature and not simply on the total amount of food eaten.

(4) Groups of rats weaned on their 17th day of age suffered a higher incidence of acute liver necrosis and had a shorter survival time than their littermates weaned on their 25th day.

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EFFECT OF DIFFERENT MAKES OF CASEIN ON THE PRODUCTION OF ACUTE DIETARY LIVER NECROSIS IN THE RAT

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From 1949 till the summer of 1951, the casein used in diets to induce acute liver necrosis in rats was the "vitamin-free" product of Glaxo Laboratories, Ltd., Middlesex, England. When this casein became unavailable commercially, "low-vitamin" content casein of Genatosan, Ltd., Loughborough, England, was substituted. The next two experiments were conducted in the autumn of 1951 under those conditions which had been found to be most favorable for the production of the disease; *i.e.*, when the rats were housed in an environmental temperature of about 75°F. and allowed to eat to appetite. In these latter experiments,[1] no rat suffered from liver necrosis. This paper reports in more detail the results obtained by a direct comparison, using littermates, of two batches of casein, Genatosan casein "low-vitamin" content, batch no. 078689, supplied in 1951, and Glaxo lactic casein "C" (unextracted), batch no. UA529, supplied in 1947. It was not possible to make a direct comparison with the sample of Glaxo "vitamin-free" casein used in previous experiments.

Methods

Two experiments lasting from February to April, 1952 and from April to July, 1952 are described. The environmental temperature of the animal house was 74°–78°F. Young male rats of the hooded Lister strain, Rowett Institute stock, were weaned on their 17th day, instead of their 24th day, as is customary in this Institute, as it had been found that groups with a higher degree of sensitivity to liver necrosis were thus obtained.[1, 2]

The *diets* contained casein 16 (Glaxo lactic casein "C" unextracted or Genatosan casein "low-vitamin" content), brewers' yeast 3 (Pharmaco-Chemical Products Ltd., London), sucrose 68, commercial lard 7, McCollum's salts 4, cod liver oil (B.P.) 2 per cent, choline chloride 200 mgm./100 gm. diet, and B vitamins but without added vitamin E. These diets were offered from the 17th to 37th day of age and were followed on the 38th day of age by diets in which the casein was reduced to 8 per cent and the sucrose increased to 76 per cent. The rats were always allowed to eat to appetite.

Groups. In experiment 1, six male rats from each of five litters were allocated at random to 3 groups, A, B, and C, on their 17th day of age. Groups A and B were given diets containing Genatosan casein "low-vitamin" content. The rats in group B were given in addition 0.5 μg. vitamin B_{12} orally three times a week from their 38th day of age. Group C was given diets containing Glaxo lactic casein "C." In experiment 2, six male rats from each of twelve litters were allocated at random to 2 groups, D and E. Three rats from each litter were put into each group, and fed on diets containing either Genatosan casein or Glaxo lactic casein ' C."

TABLE 1

INCIDENCE OF ACUTE LIVER NECROSIS IN EXPERIMENTS 1 AND 2

Expt. no.	Group	Make of casein use in diets	No. of rats tested	No. dead with acute liver necrosis	Median survival time days‡	Remarks
1	A	Genatosan* casein "low-vitamin" content	2 from each of 5 litters =10	4	—	One rat died on each of the following days after feeding experimental diet; 11, 12, 24, 28.
	B	Genatosan casein "low-vitamin" content + vit. B_{12}**	2 =10	1	—	One rat died 8 days after feeding experimental diet
	C	Glaxo† lactic casein "C" unextracted	2 =10	9	7	Two rats died on each of the following days after feeding experimental diet; 4, 5, 7: and three on the 13th day.
2	D	Genatosan casein "low-vitamin" content	3 from each of 12 litters =36	16	—	See graph
	E	Glaxo lactic casein "C" unextracted	3 =36	33	9	

* Genatosan Ltd., Loughborough, Leicestershire, England.

** Vitamin B_{12} : Cytamen (Glaxo) 0.5 μg. in 0.1 ml. water given by mouth 3 times a week from 38th day.

† Glaxo Laboratories, Ltd., Greenford, Middlesex, England.

‡ Days after feeding experimental diet; *i.e.*, days after 38th day of age. Survivors killed on 47th day in experiment 1 and on 72nd day in experiment 2.

Results and Discussion

The accompanying *table* and graph (TABLE 1) show the incidence of acute liver necrosis and the median survival time of the rats. It is obvious that batch UA529 of Glaxo lactic casein "C" induced the higher incidence of liver necrosis.

Since Genatosan casein "low-vitamin" content might be low in vitamin B_{12}, this vitamin was given to one group of ten rats (group B - TABLE 1). The addition of this vitamin may have delayed necrosis, but the numbers studied were too small for the result to be statistically significant.

In experiment 2, among the rats fed on the diet containing Glaxo lactic casein "C" (group E), 2 died from acute liver necrosis on their 36th day of age, another on its 37th, and a fourth on its 38th day; *i.e.*, before the percentage of casein in the diet was reduced from 16 to 8. György and Goldblatt,[3] in their original report on "Hepatic injury on a nutritional basis," used a diet containing 18 per cent casein (brand unspecified). Schwarz,[4] using a diet containing 15–30 per cent of his casein VI, obtained a high incidence of liver necrosis. In an earlier experiment,[5] in which a diet containing 19 per cent Glaxo "vitamin-free" casein was used, one rat among five died from acute liver necrosis. The results of experiment 2 are further evidence that a high level of protein *per se* in the diet does not necessarily prevent dietary liver necrosis.

Glaxo lactic casein "C" batch UA529 was a crude product of New Zealand

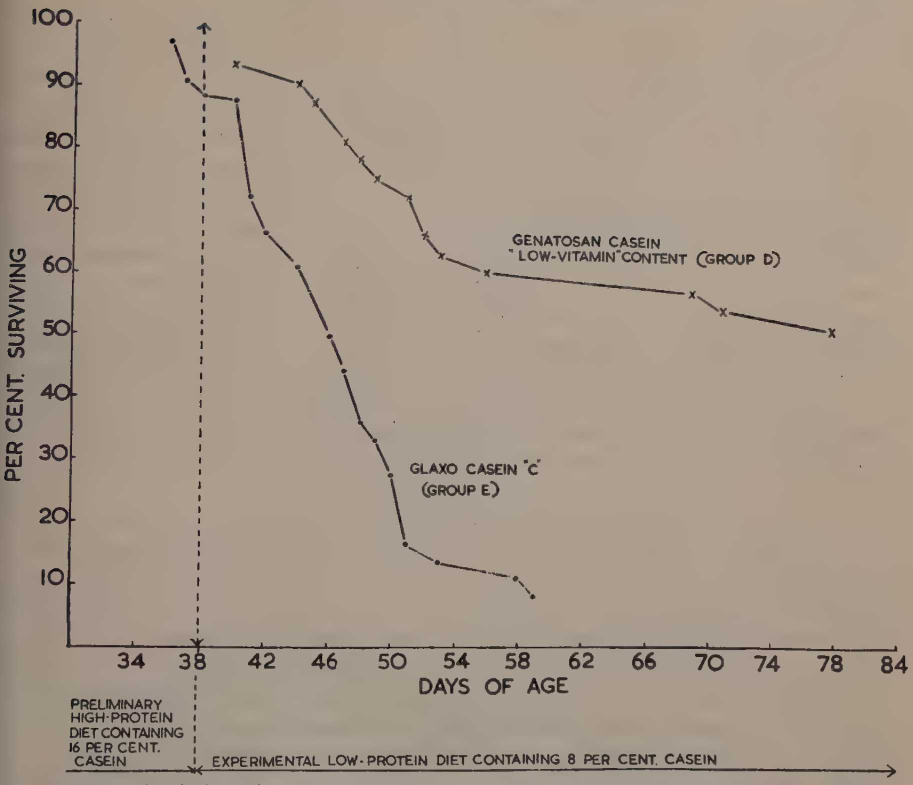

FIGURE 1. Graph of experiment 2. Percentage of rats surviving when fed on diets containing either Genatosan casein "low-vitamin" content or Glaxo casein "C." In group D (Genatosan casein) one rat died on 53rd day of age from lung hemorrhage; one rat died on 60th day of age from severe diarrhea and one rat was killed on 60th day of age. None of these rats had liver necrosis and are omitted from the calculation of percentage surviving. In group E (Glaxo casein "C") four rats died from liver necrosis before the low-protein diet was fed.

origin.[6] Genatosan casein "low-vitamin" content prepared by acid precipitation, followed by extraction with methylated spirits and ether, is usually made from British liquid milk, although reconstituted dried skim milk of New Zealand origin is sometimes added if a sufficient quantity of liquid milk is not available.[7] In two earlier experiments[2] when no liver necrosis resulted, an unnumbered batch of Genatosan casein "low-vitamin" content was used. In the experiments 1 and 2 of the present paper, batch No. 078689 of Genatosan casein "low-vitamin" content was made from a mixture of milks of British and New Zealand origin. Unfortunately, no information is now available about the unnumbered batch of the earlier experiments. The differences between caseins prepared from British or New Zealand milk may be due to age or to differences arising in preparation or to differences in the milks themselves, which may arise from differences in the feeding systems.

That caseins differ in their ability to lead to liver necrosis was first reported by Schwarz in 1944.[4] Despite this, many workers have omitted to mention the brand of casein they employ. Schwarz's casein VI which induced liver necrosis may have been regarded as specially treated casein because he prepared it from "Kasein Hammarsten Merck 2242" by repeated acid precipitation from dilute hot alkaline solution and thus purified it still further. Even when casein VI was incorporated at a level of 15 or 30 per cent of the diet, a high incidence of liver necrosis resulted. The unpurified casein Merck 2242 did not induce liver necrosis. As a result of their studies with a water-washed casein and an oxidized casein, Hove, Copeland, and Salmon[8] came to the conclusion that acute liver necrosis or massive lung hemorrhage in rats depended on a simultaneous deficiency of Vitamin E and a casein co-factor. More recently Schwarz[9, 10] has reported that a substance ("Factor 3") which prevents liver necrosis is present in a variety of natural materials including American crude casein and "vitamin free" casein prepared by repeated alcohol extraction. It should be emphasized that Glaxo lactic casein "C" (unextracted) is a crude product and, therefore, it is not possible to state without further investigation whether the difference between its liver necrogenic properties and those of Genatosan casein "low-vitamin" content is due to a difference in content of "Factor 3" or whether there is some other reason.

Summary

(1) There was a higher incidence of acute liver necrosis amongst rats housed in an environmental temperature of 74–78°F. and allowed to eat to appetite, when the casein in the diet was the lactic casein "C" (unextracted) of Glaxo Laboratories, Ltd., Middlesex, England, than when the "low vitamin" content casein of Genatosan Ltd., Loughborough England, was used.

(2) Glaxo lactic casein "C" (unextracted) is a crude product of New Zealand origin. Genatosan casein "low-vitamin" content is prepared from British liquid milk with or without the addition of reconstituted dried skim milk of New Zealand origin.

Acknowledgments

I am indebted to Dr. W. F. J. Cuthbertson of Glaxo Laboratories, Ltd., and to Mr. F. A. Todd of Genatosan Ltd., for information about the preparation of the caseins used in the experiments described in this paper.

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THE INFLUENCE OF THE ENDOCRINE GLANDS ON THE DEVELOPMENT OF ACUTE MASSIVE LIVER NECROSIS

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The first indication that the endocrine glands probably had some influence on the development of dietary liver necrosis was given in 1935, by Weichselbaum[1] in his paper on the nutritional requirement of the rat for methionine and cystine. The lethal liver lesion that was produced in his rats on a diet low in these amino acids was almost undoubtedly acute massive hepatic necrosis, although no histological description of the tissue was given by him. In this publication, he refers to the fact that the mortality was definitely less in the females. Presumably, this difference is due directly or indirectly to the action of the sex glands. Similar observations have been made by György[2] and by our group.[3] Our results, utilizing rats of average initial weight 129 gm. and in an experiment terminated after 105 days, are shown in TABLE 1. Although the mortality is essentially the same, the average time at which death occurred is significantly longer in the females.

An interesting observation has been made by Ferret[4] to the effect that diets designed to induce liver necrosis caused continuous oestrus in ovariectomized animals in which 5 mgm. estradiol had been implanted into the spleen. The addition of cystine to the diet restored the ability to inactivate the estrogen. On the other hand, alpha-tocopherol did not have a similar action; and it must be concluded, on the basis of this work at least, that the inability of animals to inactivate estrogen has a negligible significance in the pathogenesis of liver necrosis.

During an investigation of hepatic damage caused by the feeding of alkali-treated casein to rats, Schwarz[5] in 1944 noted that an extract of lipids from the adrenals inhibited the development of the lesion.

In 1951, the same worker[6] reported the results of further studies on the role of the adrenals in the development of the type of dietary massive liver necrosis currently being discussed. Proceeding on the assumption that lack of glycogenesis is one of the essential features of liver degeneration and recalling the role of adrenal cortical secretion in promoting this process, he tested the effect of cortisone on rats being fed a basal necrogenic diet low in methionine, cystine, and alpha-tocopherol.

The survival time was significantly and equally prolonged in both males and females, but there was no difference in the mortality due to massive liver damage (TABLE 2). This investigation showed clearly that cortisone ameliorated the necrogenic effect of the basal diet.

However, the assumption that imperfect liver glycogenesis is initially involved in dietary liver necrosis is not supported by some of our previous work. One of the first investigations carried out by us[7] on entering into this field was a study of progressive structural and biochemical changes occurring in the liver prior to and at the time of the acute necrotic episode. No change oc-

TABLE 1

EFFECT OF INITIAL WEIGHT AND OF SEX ON THE DEVELOPMENT OF LIVER NECROSIS

	Sex	Rats that died of necrosis or were killed when moribund		Rats killed at 105 days		Total incidence of necrosis	
		Rats	Days on diet (av. range)	No. showing signs of necrosis	No. showing no signs of necrosis	No. of rats	Per cent
Y	M	16	58 (35–97)	3	1	19/20	95
YF	F	16	79 (48–103)	—	4	16/20	80

TABLE 2

RESPONSE OF MALES AND FEMALES TO CORTISONE (NO. OF ANIMALS IN PARENTHESES)

	Average survival time (in days)	
	Males	Females
Without cortisone	41.5 (6)	50.1 (4)
With cortisone	57.0 (5)	65.2 (4)
Difference due to cortisone	15.5 days	15.1 days

From Schwarz.[6]

curred in liver glycogen levels until at the time of the acute episode when, as one would expect, the glycogen levels dropped essentially to zero. Apparently, liver glycogenesis proceeds unimpaired until signs of hepatic damage appear.

Although the nature of the effect of the thyroid hormone on the development of liver damage due to a variety of causes in both experimental animals and in humans is controversial, there is complete agreement on the nature of the effect of the thyroid secretion on the development of acute massive liver necrosis seen in rats fed diets slow in alpha-tocopherol and in the sulfur-containing amino-acids. Handler and Follis[8] first demonstrated the potentiating effect of desiccated thyroid when it was added to a basal necrogenic diet.

The diet used by these investigators contained 0.2 per cent desiccated thyroid, and caused acute liver damage in about 50 per cent of 50 gm. male rats at an average interval of 40 days compared to an incidence of 100 per cent in 70 days for similar rats on the basal diet. The diet (TABLE 3) used by us[10] contained 0.3 per cent desiccated thyroid and (TABLE 4) caused 88 per cent necrosis after an average interval of only 20 days or half the time required by the basal diet 39 days. These results are indicative of a profound effect of thyroid tissue on the development of liver damage. The effect of feeding propylthiouracil, on the other hand, is not so clear cut. With a level of 0.3 per cent propylthiouracil the rats died at an average interval of 47 days, but they showed no evidence of hepatic injury at death. It was assumed that the toxicity of the supplement was responsible for the death of the animals. Apparently, Handler and Follis had a similar experience with thiouracil but the time at which their animals died was not specified. At a level of only 0.05 per cent of propylthiouracil, its toxic effect was sufficiently low to permit three of the animals to survive the

TABLE 3

COMPOSITION OF BASAL DIET

Dietary ingredient	Per cent
Yeast (Fleischmann's active dried baker's yeast)	18
Cornstarch	69
Lard	5
Salt mixture*	3
Sugar vitamin mixture**	1
Cod liver oil (Mead Johnson Company 3000 I.U. of Vitamin A and 400 I.U. of Vitamin D per gram)	2
Celluflour	2

* Himsworth's salt mixture. (Personal communication, for details see Abell *et al.*[9])

** The sugar vitamin mixture contributed the following amounts of B vitamins per 8 gm. of food: thiamin. ydrochloride, 20 μgm.; riboflavin, 25 μgm.; pyridoxine hydrochloride, 20 μgm.; calcium pantothenate, 100 μgm e

TABLE 4

THE EFFECT OF DIETARY SUPPLEMENTS ON THE DEVELOPMENT OF LIVER NECROSIS

Dietary supplement	Rats dying of necrosis or killed in extremis		Rats dying of other causes		Total incidence of necrosis, corr'd.*	
	Number rats	Av. days on diet	Number rats	Av. days on diet	Number	%
None	9	39	1	42	9/10	90
0.3% desiccated thyroid	7	20	3	13	7/8	88
0.05% propylthiouracil	2	59	5	55	3/10**	30
0.3% potassium thyiocyanate	8	56	2	69	8/10	80
0.3% desiccated thyroid +0.5% L-cystine	0	—	8	57	0	0
0.3% desiccated thyroid +2 mgm. α-tocopherol acetate per 8 gm. diet	0	—	9	43	0	0

From McLean and Beveridge.[10]

* Rats that died of causes other than liver necrosis were eliminated from consideration if they died prior to the time at which the first death due to liver necrosis occurred in the remainder of the group. This convention had to be applied in the case of the group receiving 0.3% desiccated thyroid.

**One animal dying at 66 days showed necrosis in a small part of one lobe of the liver, and it was believed that this lesion was insufficient to have caused the death of the animal.

experiment, and of the animals that died only three showed acute liver damage. The incidence of liver necrosis was, therefore, decreased and required a longer time to develop, 59 days compared to 39 on the basal diet. These data, in our opinion, clearly indicate that propylthiouracil decreased the necrogenicity of the basal diet. Although it is likely that this effect may be explained by the antithyroid action of the drug, the possibility that the prophylactic action may be due to the provision of sulphydryl groups must be kept in mind since György *et al.*[11] have shown that this compound exists in tautomeric forms.

According to Astwood[12] there are two large classes of antithyroid agents: those that inhibit the uptake of iodine by the thyroid gland, but whose action can be readily overcome by administration of sufficient iodine; and those whose primary action is to depress the utilization of iodine in the synthesis of thyroxine. Propylthiouracil may be considered to be a member of the latter group, and it was considered worth while to test the effect of a compound belonging to the former class. Potassium thiocyanate was chosen and, at a level of 0.3

per cent, it effected a significant increase in the length of time required for death to occur, 56 days compared to 39 days for the rats on the basal diet. There was no effect, however, on the actual incidence of acute hepatic damage.

Supplementation of the basal diet with 0.5 per cent L-cystine or 2 mgm. alpha-tocopherol per 8 gm. of diet did not overcome the well-known toxic effects of excessive amounts of thyroid tissue, but the animals survived beyond the average time at which death occurred on the unsupplemented ration (indeed, three rats survived the experimental period of 105 days), and none of the rats in these groups developed massive hepatic necrosis. The fact that both alpha-tocopherol and cystine prevented liver necrosis in hyperthyroid animals led us to conclude that a high metabolic rate *per se* is not a prime factor in pathogenesis and that the level of thyroid activity is a subsidiary, not a determining factor, in the production or prevention of this hepatic lesion.

From time to time, we have observed pancreatic necrosis in some of the rats killed *in extremis* and showing acute liver damage. This finding raised the possibility that, despite the absence of any consistent obvious lesion in the pancreas, this organ might be in some way involved in the process under study. An initial pancreatic lesion might result in active proteolytic enzymes gaining the portal blood stream and causing the type of extensive liver damage under investigation here. Certainly, the intravenous administration of chymotrypsin as was shown by Tagnon *et al.* causes wide-spread damage including necrosis, not only of liver cells, but also of kidney tubules, a lesion that is also frequently seen in animals showing acute massive hepatic necrosis. The fact that the blood from the pancreas travels primarily to the left half of the liver fitted in quite nicely with the well-substantiated observation that the left half of the liver is usually the most severely damaged part. It was decided, therefore, to investigate the possible etiological role of the pancreas although it must be admitted that our thoughts were directed primarily towards the exocrine rather than the endocrine function of this gland.

As complete a pancreatectomy as possible was performed on a number of males rats. Since a splenectomy was done in this procedure, control groups of rats were comprised not only of sham-operated animals, but also a group on which splenectomies had been carried out. These animals, of average initial weight about 140 gm. were fed a basal necrogenic diet in the usual way and, after an interval of 290 days, the experiment was terminated. The results are shown in TABLE 5.

TABLE 5

EFFECT OF PARTIAL PANCREATECTOMY AND SPLENECTOMY ON THE DEVELOPMENT OF LIVER NECROSIS

Group	Number of rats showing liver necrosis	Incidence of necrosis	Days on diet of rats showing liver necrosis	
			Average	Range
Normal controls	1	1/11	68	
Depancreatized and splenectomized	3	3/10	181	143–213
Splenectomized	3	3/11	195	176–213

Although we were somewhat disturbed by the low incidence of liver necrosis, these findings show clearly that neither partial pancreatectomy plus splenectomy, nor splenectomy alone has any effect on the development of acute liver damage. On the other hand, we readily admit that there may have been sufficient pancreatic tissue left to account for the production of the liver lesion, but we regard this possibility as being remote.

In conclusion, it would appear that, although various investigations have demonstrated that secretions from the endocrine glands do influence one way or another the development of dietary liver necrosis, there is no clear-cut evidence to show that any of the endocrine secretions have a primary role in the development of this hepatic lesion.

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Discussion of the Paper

Doctor Klaus Schwarz: I should like to point out that food intake greatly influences the development of dietary necrotic liver degeneration. This variable is of primary importance in the interpretation of results such as reported by Doctor Beveridge. We have investigated, in collaboration with Doctor R. Scow of our Institute, the effect of thyroidectomy on the development of this deficiency. It was found that Sprague Dawley rats, when thyroidectomized at weaning, did not develop necrotic liver degeneration even after experimental periods extended long beyond the time of death of all the littermate controls fed *ad libitum*. Realizing, however, that rats fed *ad libitum* do not constitute proper controls for others which do not eat due to some endocrine disturbance, we have run simultaneous, unoperated littermate controls which were *pairfed to the thyroidectomized group*. These animals, which received approximately 50 per cent of the diet consumed by the *ad libitum* fed group, survived as well as the thyroidectomized ones. It may be possible that we have to interpret all the reported effects of decreased or increased thyroid activity on necrotic liver degeneration as resulting from decreased or increased food consumption, and not from the endocrine disturbance directly.

FACTORS PROTECTING AGAINST DIETARY NECROTIC LIVER DEGENERATION

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Introduction

It is a pertinent characteristic of every natural phenomenon that, in addition to its primary cause, secondary conditioning causes influence its origination. This is certainly true for all deficiency diseases. In the preceding papers, various nonspecific factors affecting the development of dietary necrotic liver degeneration in the rat have been presented and discussed. The present paper is devoted to the factors which are primarily involved.

The development of necrotic liver degeneration has been shown to depend on such complicating circumstances as the over-all composition of the deficient diet, the endocrine status of the animal, its daily food allowance, and its environmental temperature. No matter how profoundly these various influences either enhance or inhibit the process, the fact remains that dietary necrotic liver degeneration is, in the first place, a deficiency disease. It can be produced experimentally by diets which lack certain essential nutrients and it can be prevented by these dietary components. The disease is different, however, from many other deficiency diseases in that it does not result from the lack of one single dietary factor. It is more complicated. At least three different nutritional factors must be lacking simultaneously before the disease develops. This complexity has prevented a quick solution of the many problems which are presented by the deficiency. It also makes dietary necrotic liver degeneration especially interesting from a biochemical and metabolic point of view. Like fatty liver and cirrhosis, the other liver disease produced by dietary means, dietary necrotic liver degeneration is of multiple origin; but the nutritional factors involved are different. Substances which prevent the first syndrome can even enhance the development of the second, and vice versa.[1]

The three nutritional factors at present clearly separable and recognized as protecting against dietary necrotic liver degeneration are cystine, vitamin E, and Factor 3. The action of cystine was detected in 1935 by Weichselbaum,[2] and later by Daft, Sebrell, and Lillie;[3] it was confirmed by Hock and Fink,[4] and many others. The role of vitamin E as a protective agent was elucidated between 1941 and 1944 by Schwarz,[5, 6*] and has been confirmed by György and collaborators[7] and by various other groups between 1947 and 1950. Finally, the existence of Factor 3 has been established in this laboratory only a few years ago.[8] An account of this work is given below.

Diets for the Production of Dietary Necrotic Liver Degeneration

Rations designed to produce dietary necrotic liver degeneration must be low in cystine, free from vitamin E and, furthermore, deficient in Factor 3, since

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[*] Printed and published in 1945 in the United States under the auspices of U.S.O.A.P.C., by J. W. Edwards, Ann Arbor, Mich.

each one of these three different substances can afford protection by itself. A variety of diets which fulfill these requirements more or less satisfactorily and which have been observed to induce necrotic liver degeneration in rats is shown in TABLE 1.

It is debatable whether liver necrosis produced by these different rations is really a single disease entity, or whether various kinds of types or liver necrosis have to be distinguished. Only future research can clarify this point.

The majority of the rations listed are not only deficient in the three factors protecting against the disease, but also inadequate in other dietary essentials; for example, in amino acids. Animals kept on such diets suffer from a variety of deficiencies, their food intake is low, and liver necrosis may be seen in only a small percentage of them after rather extended experimental periods.

The author's experience with dietary necrotic liver degeneration dates back to 1941, when a method for the production of the disease in rats was discovered. An extremely purified casein, so-called casein VI, was used. The disease was produced regularly and at will in large numbers of animals. This procedure was put to use as a test method for the concentration and identification of liver-protecting substances. When properly supplemented with the other well-recognized vitamins, the casein VI diet produced a high incidence of necrotic liver degeneration in a rather short period of time. In the series of experiments from 1941 to 1945 with the 15 per cent casein VI diet, an average surivival time of 42 days and an incidence of 84 per cent of liver necrosis was observed.[5]

In the present experiments since 1949, using a 30 per cent Torula yeast diet, the survival time averages approximately 45 days and the incidence of necrotic liver degeneration reaches 100 per cent.[9] In the following, results from both these series are employed to clarify essential aspects of factors protecting against the disease. The composition of the two basal diets used is presented in TABLE 2.*

Comparison of Various Sulfur-Containing Amino Acids

One aspect which requires accurate investigation is the activity of cystine when supplemented to the casein VI diet. No protective action was observed when 40 mgm. of L-cystine was given as daily supplement in a separate cup to animals kept on the casein VI ration.[5] From more recent work, particularly that of Geiger,[13] it has become apparent that it may be important to administer the amino acid simultaneously with the diet in order to assure its utilization. When cystine is added to the Torula ration, the amount required for protection against dietary necrotic liver degeneration is comparatively high. Supplementation of 0.1 per cent has only a small effect. With 0.2 per cent, approximately 75 per cent protection is afforded. Furthermore, much higher levels (for example, 5 per cent or 1 per cent), do not prevent the disease in all animals.[9]

We have investigated rather thoroughly the relationship between the various

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* It must be mentioned that attempts in other laboratories, as well as in our own, to produce casein VI from the "vitamin-free" caseins available on the market have been unsuccessful.[10, 11, 12] "Vitamin-free" caseins are mostly prepared by continuous extraction with hot methanol, while the older experiments were made with a "Hammarsten" casein (Merck, Darmstadt, No. 2242) which had been repeatedly precipitated with acid from alkaline solutions.

TABLE 1

DIETS PRODUCING NECROTIC LIVER DEGENERATION IN RATS

Cystine-free (corn starch 80%; dry milk powder 16%)	Weichselbaum[2]
Cystine-free (amino acids in crystalline form)	DuVigneaud, Dyer, and Kies[24] Glynn, Himsworth, and Neuberger[16]
Low casein (10% or less)	Daft, Sebrell, and Lillie[3] György and Goldblatt[11] Hove and Harris[25] Schwarz[5]
Casein VI (alkali-treated, 15%)	Hock and Fink[4]
Yeast	Glynn and Himsworth[15] Schwarz[8, 20] György and Goldblatt[26] Schwarz[27]
High cod liver oil	
Potato protein Pea protein Cereal protein Gelatin in various combinations	Hock and Fink[28]
Soybean meal (raw, fat extracted)	Matet, Matet, and Fridenson[29]
Soybean protein	Olson[30]

TABLE 2

COMPOSITION OF DIETS

Casein VI diet	Parts	*Torula* yeast diet	%
Casein VI*	15	Torula yeast	30
Butter fat (extracted)*	5**	Lard, Vit.-E-free††	5
Sucrose	65	Sucrose	59
Salts***	5	Salts‡	5
Vitamins supplemented separately†		Vitamin powder‡‡	1

* Preparation described in Schwarz.[5]

** In earlier experiments 10 parts.

*** For composition, see Schwarz.[5, 9]

† See Schwarz.[5]

†† Vitamin-E-free animal fat, stripped by molecular distillation (Distillation Products Division, Rochester, New York).

‡ Composition same as salts in Casein VI diet; since April, 1951, Hubbel, Mendel, and Wakeman. J. Nutrition. 14: 273 (1937).

‡‡ Composition, see Schwarz.[9]

sulfur amino acids in respect to dietary necrotic liver degeneration. It is generally accepted that methionine and not cystine is the sulfur-containing amino acid essential for growth and for well-being of animals. It is quite evident that the rat forms cystine from methionine, whereas it is unable to form methionine from cystine. The requirement for sulfur amino acids can be satisfied by methionine alone but not by cystine.[14] In dietary necrotic liver degeneration, on the other hand, we have a case where cystine is more important than methionine, at least in the survival of the animal. Cystine affords protection, whereas methionine is much less effective. This was discovered in 1935 by Weichselbaum[2] and was later demonstrated by Himsworth and collaborators, who at first considered necrotic liver degeneration to be a methionine deficiency[15] and then upon more careful investigation, asserted that

TABLE 3
COMPARISON OF PROTECTIVE EFFECTS OF L-CYSTINE AND L-METHIONINE AGAINST DIETARY NECROTIC LIVER DEGENERATION

Supplement	%	% protection*
L-cystine	0.1	17
L-methionine	0.123	3
L-cystine	0.2	68
L-methionine	0.246	12

* Per cent protection = per cent reduction of reciprocal survival time as compared to littermate contro group.

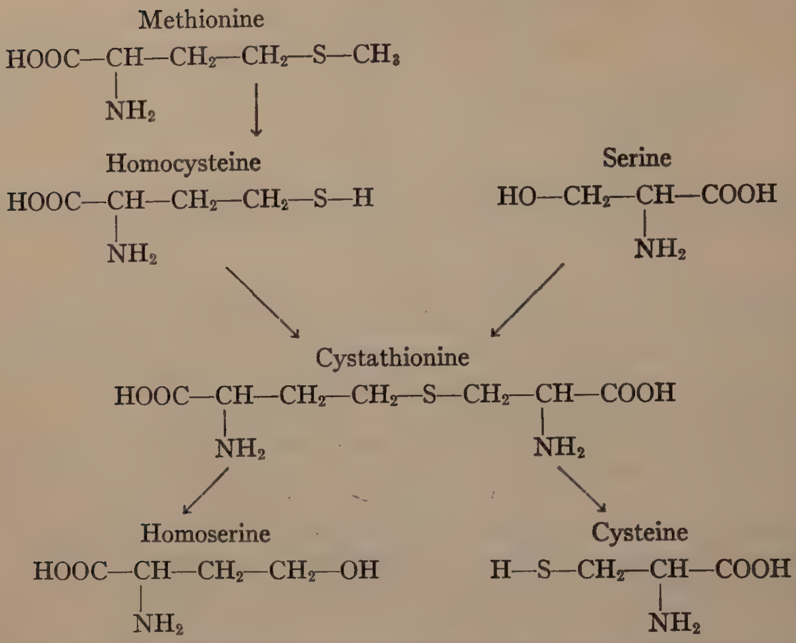
not methionine, but rather cystine, is the protective agent.[16] When L-cystine and L-methionine are compared at different dose levels in the Torula diet, it is seen that supplements of L-methionine are not entirely ineffective. They show usually about 25–35 per cent of the activity of equivalent amounts of L-cystine. A representative experiment is given in TABLE 3.

Teleologically, it could be theorized from these results that, in an animal succumbing to liver necrosis, the transformation of methionine to cystine is not sufficient to prevent death. If this were a metabolic insufficiency, it could be due either to an increased demand for cystine exceeding the natural tendency and capacity to perform this conversion, or to a real inhibition of the transformation by a metabolic block which could be caused by lack of vitamin E and Factor 3. It is conceivable, but requires further elucidation, that the methionine-cystine transformation occupies a key position in the antagonistic relationship between the factors which protect against dietary necrotic liver degeneration (prevented by cystine) on the one hand, and the factors which protect against fatty liver and cirrhosis (prevented by methyl groups) on the other. The pathway of the transformation, as it is understood at present, involves the main reactions found on the following page.

In an attempt to detect which step of the transformation is inadequately performed, various intermediates were fed. DL-homocystine was as ineffective as methionine. Cystathionine was not tested. The last step in the conversion of methionine to cystine is the oxidation of cysteine to cystine. It was found, to our surprise, in repeated experiments that L-cysteine when fed either by stomach tube or when given in the diet does not replace L-cystine. The activity of cysteine is as low as that of methionine (TABLE 4).

From these results, a number of essential questions can be derived: One might ask whether cysteine is as easily converted to cystine in normal intermediary metabolism, as is usually taken for granted, or whether in animals on necrosis-producing diets this oxidative process is especially defective. The question also arises whether vitamin E or Factor 3 participates catalytically in this reaction or in some other closely associated reaction of sulfur amino acid metabolism. Furthermore, it becomes a question as to whether the effect of cystine on dietary necrotic liver degeneration is strictly a physiological-nutritional one or whether cystine in this instance exerts some "pharmacological" action in excess of its normal biological functions and in excess of the normal cystine requirement. It deserves attention that the effect of cystine

Methionine-Cystine Transformation



Methionine

$HOOC—\underset{NH_2}{CH}—CH_2—CH_2—S—CH_3$

Homocysteine

$HOOC—\underset{NH_2}{CH}—CH_2—CH_2—S—H$

Serine

$HO—CH_2—\underset{NH_2}{CH}—COOH$

Cystathionine

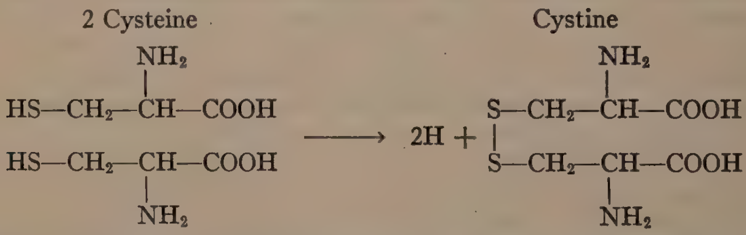
$HOOC—\underset{NH_2}{CH}—CH_2—CH_2—S—CH_2—\underset{NH_2}{CH}—COOH$

Homoserine

$HOOC—\underset{NH_2}{CH}—CH_2—CH_2—OH$

Cysteine

$H—S—CH_2—\underset{NH_2}{CH}—COOH$



2 Cysteine

Cystine

$$\begin{array}{l} HS—CH_2—\overset{NH_2}{CH}—COOH \\ HS—CH_2—\underset{NH_2}{CH}—COOH \end{array} \longrightarrow 2H + \begin{array}{l} S—CH_2—\overset{NH_2}{CH}—COOH \\ S—CH_2—\underset{NH_2}{CH}—COOH \end{array}$$

is obviously not due to a known "detoxification" mechanism for the simple reason that in detoxification reactions cysteine is as active as cystine.[17]

Vitamin E as a Protecting Agent

The work which led to the identification of vitamin E as a protective factor in dietary necrotic liver degeneration was described in detail in 1944.[6] Suffice it to mention here that, in 1941, it was recognized that wheat germ exerted a strong protective action against necrotic liver degeneration in rats on casein VI diets and that, at that time, isolation of the active principle was initiated. After two years, highly purified fractions of the factor from the wheat germ were obtained. The best concentrates contained up to 44 per cent of a substance which was shown to be vitamin E. Early in 1943, synthetic α-tocopherol acetate was found to be active.

From a series of tests performed in 1943, the daily dose of synthetic α-tocopherol acetate required for 50 per cent protection was found to be 67 γ. Using

TABLE 4

COMPARISON OF L-CYSTINE AND L-CYSTEINE (SUPPLEMENTATION STARTED ON THE 25TH DAY)

Supplement	%	% protection*
L-cystine	0.5	96.6
L-cysteine	0.5	16.5
L-cystine	1.0	97
L-cysteine	1.0	26.2

* Per cent protection = per cent reduction of reciprocal survival time as compared to littermate control group.

the Torula yeast diet and Sprague-Dawley rats in Bethesda in 1950, we found that about 50 γ are required daily for the same effect. In the later experiments, the vitamin was added to the diet. These levels of vitamin E required were, in the earlier publications, considered to be much higher than the normal vitamin E requirement. Dietary necrotic liver degeneration was interpreted as a "relative" vitamin E deficiency.[6] In view of more recent data, however, it appears that these quantities of vitamin E are actually well within the range of the normal vitamin E requirement for the prevention of deficiency symptoms in rats. It has, for example, been reported that between 30 and 70 γ of synthetic α-tocopherol acetate are required daily for maintenance of normal seminiferous epithelium in growing rats.[18] In the light of these observations, dietary necrotic liver degeneration appears to be a form of vitamin E deficiency which is modified by the simultaneous absence of Factor 3. The occurrence of dietary necrotic liver degeneration as a vitamin E deficiency indicates strongly that tocopherol has functions which are of basic importance for normal intermediary metabolism.

Factor 3 against Dietary Necrotic Liver Degeneration

An attempt was made in 1943–1944 to determine whether the procedure used for the preparation of casein VI from "Hammarsten" casein caused a "toxic" component to be formed or whether a protective factor was removed. Evidence for the first possibility could not be found, and some evidence for the later was obtained. Since "Hammersten" casein, from which the casein VI was produced, is suitable for the production of vitamin E deficiencies, the protective substance removed could be assumed to be different from tocopherol. Analyses for cystine and methionine showed that the difference between the two caseins could not be ascribed to differences in cystine or methionine (TABLE 5).

The reasoning which led to the discovery of Factor 3 may be further illustrated by the following example. Two diets are listed in TABLE 6. One is a conventional 15 per cent "vitamin-free" casein diet, the other a 30 per cent Torula ration (approximately 14 per cent protein). The first does not produce dietary liver necrosis. The other one, prepared with the same dietary ingredients except for the substitution of Torula yeast at the expense of casein and of sucrose, produces the damage with 100 per cent incidence. When the ingredients used in this experiment were analyzed colorimetrically and microbiologically for cystine and methionine, it became evident that there was no profound

TABLE 5
S-AMINO ACIDS IN CASEIN DIETS

	Cystine	Methionine	Incidence of dietary necrotic liver degeneration
	%	%	%
Casein Hammarsten diet (15%)	0.06	0.52	0
Casein VI diet (15%)	0.00	0.54	84

TABLE 6
S-AMINO ACIDS IN TORULA DIET

	Cystine	Methionine	Incidence of dietary necrotic liver degeneration
	%	%	%
Control diet, "Vitamin-free" casein (15%)	0.075	0.45	0
Torula yeast diet (30%)	0.29	0.38	100

and decisive difference in sulfur-containing amino acids. The Torula diet was even somewhat superior in cystine, but the differences were altogether so small that they were, in view of the relatively high levels of cystine and methionine required for protection, not of significance. Accordingly, the fundamental dissimilarity between these two rations in regard to liver necrosis could not be attributed to differences in cystine or methionine. Moreover, the dissimilarity could not be caused by a difference in the vitamin E contents, since both rations were apparently practically free from tocopherol. Both "vitamin-free" casein, on the one hand, and dried yeast, on the other, are standard ingredients of diets producing vitamin-E-deficiencies of the conventional type. From these considerations, it became evident that the difference between the two diets could be expected to be due to some other factor, and it seemed logical to test the "vitamin-free" casein for liver protecting activity in the Torula assay (FIGURE 1). Addition of 3 per cent of the "vitamin-free" casein produced a reduction of 66 per cent in reciprocal survival time. By comparison, an amino acid mixture simulating the composition of the casein[19] was without significant effect.

The detection of Factor 3 was facilitated by a peculiar discrepancy between European and American yeasts. In vitamin-E-free diets, almost all European dried yeasts led to the deficiency[20] whereas almost all American yeasts, particularly American brewers' yeasts, did not produce the disease.[9, 21] However, an American Torula yeast which is grown commercially on a rather simple medium was found, in 1949, to produce the deficiency with 100 per cent incidence.[9] Since that time, the 30 per cent Torula diet has been used in our laboratory in extensive investigations in attempts to concentrate and identify Factor 3. TABLE 7 demonstrates the presence of Factor 3 in an American brewers' yeast.[8] So far, no true American brewers' yeast has been encountered which did not offer protection when added to the Torula ration. This explains satisfactorily

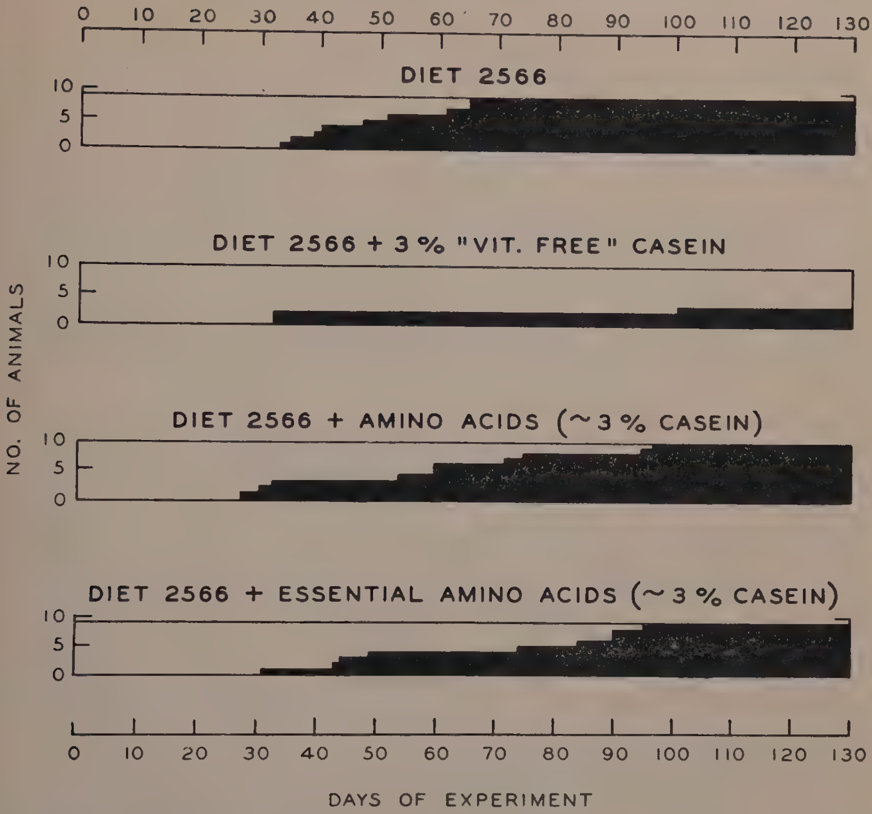


FIGURE 1. Prevention of death from dietary liver degeneration by 3 per cent casein. (Black areas: Animals dead from necrotic liver degeneration.)

why the disease could not be produced in numerous attempts with diets based mainly on these yeasts.

As starting material for the purification for Factor 3, a variety of natural sources can be used. Factor 3 has, in common with most other essential accessory foodstuffs, the property of being strongly bound to proteins. It cannot be liberated by ordinary extraction procedures. Therefore, most of the sources of Factor 3 have to be acid hydrolyzed before the fractionation of the active principle can begin. Acid hydrolysis does not alter the total Factor 3 activity, since the active substance is quite stable against this treatment. Factor 3 is found in free form in enzymatic protein digests. A commercial casein preparation has been used for the large scale purification of the active substance. The first three steps of our method have been described elsewhere.[22] The active principle can be adsorbed from crude casein digests by charcoal at slightly alkaline pH. From the absorbate, Factor 3 can be eluted by hot ethanol. After evaporating the alcohol and taking up in a small volume of water, the eluate is treated with ethanol. In this way, inactive material, mostly peptides, is precipitated. The alcohol filtrate is evaporated to dryness and then taken up on

TABLE 7

PROTECTIVE EFFECT OF YEAST K

Torula yeast %	Suppelemtn	No. of A animals	Dead due to liver degeneration	Avg. of reciprocal survival time (× 100)	% protection*
30	—	9	9	2.40 ± .18	—
27	Yeast K 3%	10	2	.58 ± .25	76
25	Yeast K 5%	8	0	.00 ± .0	100

* Per cent protection = per cent reduction of reciprocal survival time as compared to littermate control group.

a small volume of water. From this fraction, a heavy precipitate is formed upon standing in the cold, which contains most of the Factor 3 activity. Further steps of purification involve treatment with solvents like ethanol, butanol, and phenol, and also precipitation reactions with phosphotungstic acid and barium hydroxide. In this manner, fractions can be obtained from casein which protect the animals from dietary necrotic liver degeneration when given in very small amounts.

It is not possible at the moment to say very much about the chemical characteristics of the active substance. Factor 3 is quite stable against oxidizing agents, for example, H_2O_2 and ferric chloride. It is probably not an amino acid, since the customary reactions known to destroy these substances do not inactivate our fractions. Factor 3 can be expected to have a rather low molecular weight. It might well be that it is identical with a compound which is already known, though a large number of substances occurring in nature have been tested and found to be inactive.

Metabolic Interrelations in Dietary Necrotic Liver Degeneration

The peculiar fact that, in dietary necrotic liver degeneration, three substances are protective which belong to entirely different chemical groups has caused a certain difficulty in understanding the relationships involved. What the possible chemical relation between cystine and vitamin E, on one hand, and Factor 3, on the other hand, may be remains to be clarified. The exact quantitative proportions between these substances have to be elaborated when all of them are available in pure form. Considering the fact, however, that between 400 to 800 molecules of cystine are required to produce as much protection as that offered by one molecule of α-tocopherol, it could be postulated that the role of vitamin E is of a catalytic nature whereas cystine or a cystine derivative functions as a substrate.[22] From the dosage levels of Factor 3 required to obtain comparable degrees of protection, it can be suggested that Factor 3 might well belong to the group of substances which have catalytic functions in the animal organism. Both cystine and vitamin E can participate readily in oxidation reduction reactions. From preliminary experiments, Factor 3 appears to have the same property,[10] and it seems to be possible that all three of these essential dietary constituents have functions in the liver which are metabolically closely related or associated. Such a situation would explain the dietary relationship described.

An attempt has been made to locate the area of intermediary metabolism which is defective in livers of animals on necrosis-producing diets. The fact that cystine is so much more efficient than cysteine in the prevention of the defect could be interpreted as an indication of a deficiency in oxidative functions. Other observations lead to the same conclusion. At the terminal stage of the disease, lactic acid and pyruvic acid are accumulated in the blood.[10]

In preliminary experiments, it was found that the citric acid level in the liver of animals on the degeneration-producing diet was greatly increased upon injection of Na-monofluoroacetate. For weeks prior to succumbing to liver necrosis, animals on necrogenic diets show a serious incompatibility for fat.[10] These and other findings make it conceivable that the area of the primary metabolic breakdown in dietary necrotic liver degeneration is in the citric acid cycle or in some closely related function.

Summary

Three different nutritional factors have been recognized to be specifically connected with dietary necrotic liver degeneration: cystine, vitamin E, and Factor 3. Diets for the production of the deficiency in rats must be low in cystine and deficient in vitamin E and in Factor 3. Two rations fulfilling these requirements are described: the casein VI diet, used between 1940–1945; and the 30 per cent Torula yeast ration, in use since 1949.

In the prevention of dietary necrotic liver degeneration, cystine is required in rather large amounts (0.2–1 per cent in the diet). Other S-containing amino acids, like methionine, homocystine and also cysteine are only approximately one third as effective as cystine.

α-Tocopherol acetate was found to afford 50 per cent protection at 67 γ daily per animal in the old series and about 50 γ daily in the new series. These levels are within the range of the normal vitamin E requirement of the rat.

The detection of Factor 3, its occurrence in various caseins and in brewers' yeast, and the method of purification of Factor 3 concentrates are described. Factor 3 is of low molecular weight, stable against acid hydrolysis, water soluble, and apparently not identical with any of the presently well-known vitamins or amino acids.

The possible metabolic interrelations between these factors and also the metabolic aspects of dietary necrotic liver degeneration are discussed. From preliminary results it appears conceivable that there is a defect of oxidative functions in the liver undergoing necrotic liver degeneration, and that the primary metabolic defect is closely related to the citric acid cycle.

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ENZYME ABNORMALITIES ASSOCIATED WITH DIETARY NECROTIC LIVER DEGENERATION IN RATS*

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Although acute massive hepatic necrosis in the rat has been shown to be a manifestation of malnutrition, its exact biochemical pathogenesis is unknown. Frank tissue necrosis is generally assumed to be preceded by single or multiple biochemical lesions, and it seems probable that this particular syndrome of hepatic necrosis is no exception to the rule. The fact that a deficiency of one or more dietary nutrients appears to be related in a causal manner to this syndrome was of particular interest to us, and led us to undertake a study of its biochemical evolution. It was hoped, in addition, that these studies would contribute to the elucidation of the interrelationships between the sulfur amino acids and vitamin E, both of which are protective against this disease. Our initial approach to this problem was to compare the content of selected enzymes and coenzymes in the livers of rats in which hepatic necrosis was produced with others which were protected by suitable dietary supplements. The results, though preliminary, are consistent with the view that acute massive hepatic necrosis in the rat is associated with differential disruption of the citric acid cycle and depressed tissue levels of coenzyme A.

Male weanling rats of the Sprague-Dawley strain were used in these experiments. One group was fed the basal diet composed basically of soy protein and lard and supplemented with crystalline vitamins as shown in TABLE 1. This diet supplied about 0.2 per cent methionine and was essentially devoid of cystine and Vitamin E. Additional groups were fed the basal diet plus protective supplements. These included Vitamin E alone, cystine alone, cystine plus Vitamin E, methionine alone, and methionine plus vitamin E. Vitamin E was administered at the level of 3 mgm. of α-tocopherol twice weekly by dropper and the sulfur amino acids were included at the level of 0.5 per cent. The growth curves of these animals are compared with that obtained in rats fed a diet containing 18 per cent casein in FIGURE 1. No growth was obtained with either the basal diet or the basal diet plus vitamin E. Other diets stimulated intermediate growth rates between that observed on the basal diets and the diet containing 18 per cent casein.

After 4 to 6 weeks, animals on the basal diet developed lesions typical of acute massive hepatic necrosis both grossly and microscopically.[1] No lesions were observed in the rats receiving vitamin E or methionine. Occasional microscopic lesions were found in animals receiving only cystine supplements to the basal diet listed in TABLE 1. In the absence of vitamin B_{12}, the incidence of necrosis in rats receiving cystine alone appeared higher.

At the time of the first deaths in the basal group, rats were taken from all groups for the determination of hepatic pyruvic oxidase, succinoxidase, trans-

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* Supported in part by grants-in-aid from the Nutrition Foundation, Inc., New York, N. Y., and the Williams-Waterman-Fund of the Research Corporation, New York, N. Y.

TABLE 1
BASAL DIET

	Per cent
Alpha protein	15.0
Sucrose	72.8
Lard	6.0
Salts (H.M.W.)	4.0
Cod liver oil	2.0
Choline	0.2

B-vitamins were added in the following amounts expressed as mgm. per kgm. diet: Thiamine 5.0, riboflavin 5.0, pyridoxine 5.0, calcium pantothenate 20.0, niacin 40.0, folic acid 1.5, and biotin 0.05. Menadione was added at the level of 0.08 mgm./kgm.

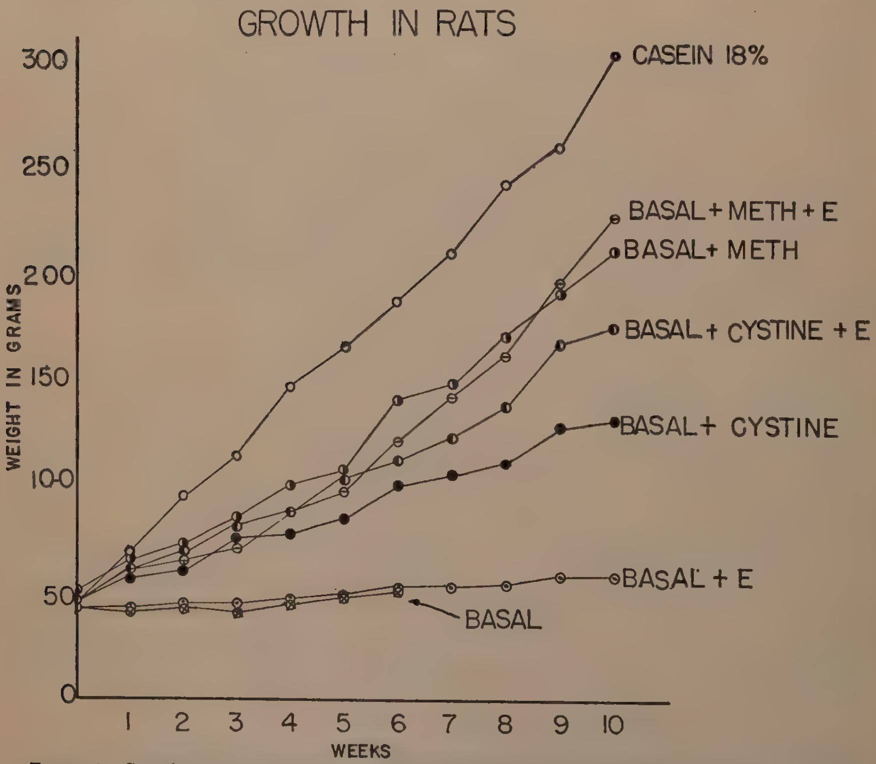

FIGURE 1. Growth rates of weanling rats fed the experimental diets and a diet containing 18 per cent casein over a 10-week period.

aminase, and xanthine oxidase. These particular enzymes were chosen for assay in order to gain as much information as possible about the carbohydrate, amino acid, and purine metabolism of necrotic and protected livers. The animals were killed by decapitation and 10 per cent homogenates of liver in isotonic KCl were prepared. Appropriate amounts of homogenate were taken for each of the assays.

The term pyruvic oxidase is used in this paper in its broadest sense of includ-

TABLE 2

PYRUVATE OXIDATION IN HOMOGENATES OF LIVER FROM NECROTIC, NON-NECROTIC-DEFICIENT, VITAMIN-E- AND SULFUR-AMINO-ACID-TREATED RATS

Diet	Rats nr.	Necrosis gross	Q_{O_2} none	Q_{O_2} pyruvate	Q_{O_2} pyruvate + ATP
Basal	9	+	1.0 ± 0.1	1.5 ± 0.3	3.1 ± 0.6
Basal	5	0	2.2 ± 0.6	2.8 ± 0.4	7.2 ± 0.9
Basal + E	6	0	2.9 ± 0.6	3.2 ± 0.8	6.9 ± 0.9
Basal + cystine	5	0	3.2 ± 0.7	4.8 ± 0.5	6.0 ± 0.9
Basal + cystine + E	5	0	4.0 ± 0.4	5.7 ± 0.7	8.9 ± 1.3
Basal + meth.	4	0	3.3 ± 1.0	3.2 ± 1.1	7.9 ± 0.8
Basal + meth. + E	5	0	3.1 ± 0.4	4.2 ± 0.4	8.8 ± 0.8

ing all oxidative reactions leading to the complete combustion of pyruvate by a tissue homogenate.[2] The assay was carried out in the conventional Warburg apparatus at 37°C. Oxygen consumption was measured in each of three flasks containing, respectively, 0.5 ml. of homogenate plus 2.5 ml. of phosphate-saline buffer with no added substrate, with sodium pyruvate added at 5 mM/liter; and with pyruvate at 5 mM/L., plus ATP at 1 mM/L. The addition of ATP to the last flask permitted the observation of pyruvate oxidation under conditions which tended to prevent deactivation of tricarboxylic acid cycle intermediates and the fission of coenzymes. Succinoxidase was determined by the method of Schneider and Potter,[3] transaminase by the method of Ames and Elvehjem,[4] and xanthine oxidase by the method of Axelrod and Elvehjem.[5] All substrates were tipped in at "zero" time. The units for the pyruvic oxidase assay are given as Q_{O_2}, calculated for a 20-minute period, and the units for the other assays are those used by their respective authors.

The results obtained with the pyruvic oxidase assay are shown in TABLE 2. The animals on the basal diet were divided into two groups depending upon the presence or absence of gross necrosis. It can be seen that both the endogenous O_2 uptake and the oxidation of pyruvate, with or without added ATP, was markedly reduced in homogenates of liver from rats showing gross necrosis. The activity of the control group (rats fed basal plus methionine plus vitamin E) was roughly three times as great in comparable flasks. Livers from animals on a deficient diet not showing necrosis showed diminished oxygen uptake with added pyruvate alone but with added ATP were essentially normal. Livers from groups fed the basal diet plus vitamin E were intermediate between the rats on the basal diet not showing necrosis and the controls, although not significantly different from either. None of the rats on other protective regimens showed values significantly different from the controls.

TABLE 3 presents the results of the assays for succinoxidase, transaminase, and xanthine oxidase in these same groups of animals. It is noteworthy that the activity of succinoxidase and transaminase are virtually the same in all groups. The liver succinoxidase activity of the deficient group with necrosis is statistically just less than that of supplemented controls, although not different from the deficient animals not showing necrosis. The change is minute in comparison with the drop in pyruvic oxidase activity. It seemed remarkable to us that hepatic transaminase activity was maintained in the severely

TABLE 3

SUCCINOXIDASE, TRANSAMINASE, AND XANTHINE OXIDASE ACTIVITIES OF LIVER FROM NECROTIC, NON-NECROTIC-DEFICIENT, VITAMIN E- AND SULFUR AMINO ACID-TREATED-RATS

Diet	Rats no.	Necrosis gross	Succinoxidase	Trans-aminase	Xanthine oxidase
Basal	9	+	31 ± 3	64 ± 2	39 ± 9
Basal	5	0	38 ± 4	55 ± 5	13 ± 9
Basal + E	6	0	39 ± 3	60 ± 4	6 ± 5
Basal + cystine	5	0	39 ± 2	57 ± 4	137 ± 8
Basal + cystine + E	5	0	47 ± 5	65 ± 9	20 ± 13
Basal + meth.	4	0	40 ± 2	63 ± 11	160 ± 10
Basal + meth. + E	5	0	42 ± 2	62 ± 8	138 ± 8

necrotic group, particularly since it has been reported that certain manifestations of pyridoxine deficiency appear in association with vitamin E deficiency in rabbits.[6-7]

The xanthine oxidase activity of the livers from animals in this study were of great interest. The lowest values were obtained in rats fed the basal diet plus vitamin E. This was probably due to the poor biological value of the soy protein, since it has been reported[8] that protein deprivation alone lowers liver xanthine oxidase activity. It was of interest that the animals with hepatic necrosis on the basal diet, which was not only low in organic sulfur but also devoid of vitamin E, showed a significantly higher titer of liver xanthine oxidase. This effect of vitamin E lack mirrors an effect seen in rabbits deprived of vitamin E, in which the hepatic xanthine oxidase becomes markedly elevated.[9] The tendency of vitamin E to depress the liver xanthine oxidase is also demonstrated by a comparison of the liver xanthine oxidase values of the groups given cystine alone and cystine plus vitamin E (TABLE 3). When methionine is the supplement, the vitamin E effect is abolished. Whether this vitamin E effect is dependent upon adequate amounts of molybdenum (which is abundant in soy protein) in the diet,[10-11] remains to be determined. If methionine is required for the elaboration of xanthine oxidase in the liver, these effects of vitamin E might be explained on the basis of a decreased utilization of methionine for other purposes in vitamin E deficiency. The effect of cystine would be interpreted as a sparing action.

These contrasting changes in enzyme activity which occurred with acute hepatic necrosis in rats suggest that the "death" of a cell and the disorganization of the intracellular contents histopathologically do not necessarily mean a decline of the activity of all the cellular enzymes. Some enzyme activities, in addition to catabolic ones known to increase with cell lysis such as the cathepsins, desamidases, phosphatases, and nucleases, may even increase. How much of this change can be traced to the underlying pathological process preceding necrosis, and how much is a concomitant of necrosis *per se*? The disruption of some portion of the citric acid cycle exclusive of the succinoxidase system seemed out of proportion to the other changes, and it was thought profitable to explore the status of certain catalysts in that sequence of reactions. There were obviously many possibilities. The observation that the

TABLE 4

CONTENT OF COENZYME A IN LIVER FROM NECROTIC, NON-NECROTIC-DEFICIENT, VITAMIN-E-TREATED, AND CONTROL RATS

Diet	Rats nr.	Necrosis gross	Coenzyme A units/gm.
Basal	9	+	37 ± 5
Basal	5	0	68 ± 9
Basal + E	4	0	92 ± 8
Control	4	0	180 ± 20

rats on the basal diet developed the "porphyrin whiskers" usually associated with pantothenic acid deficiency led us to determine the rates of acetylation of para-aminobenzoic acid in intact deficient rats. The finding that these were markedly depressed in the animals with liver necrosis and moderately depressed in deficient animals without necrosis[12] coupled with the realization that coenzyme A, the active form of pantothenic acid in tissues and the bearer of the C_2-fragment, contained a sulfur moiety[13] not unrelated to cystine, stimulated us to make direct determinations of coenzyme A in livers from these animals. The technique of assay was that of Kaplan and Lipmann[14] and the results are shown in TABLE 4.

The rats having liver necrosis had markedly depressed values for hepatic coenzyme A. Those on the deficient diet not showing necrosis had levels somewhat higher, but even these were as low as ordinarily produced by pantothenic acid restriction alone.[15] Those rats protected against necrosis with vitamin E had still higher values and control rats fed either chow or the 18 per cent casein diet had high values which were similar to other normal rats examined in the past. Because of this relationship between coenzyme A content and the suspected degree of hepatic disability in this dietary disease, it is tempting to suggest an etiological relationship between depressed coenzyme levels, disruption of pyruvate oxidation, and hepatic necrosis. There are many questions which have to be answered, however, before this is a tenable hypothesis.

Is the depression of coenzyme A in necrotic liver tissue specific for this dietary deficiency disease, or does it represent a generalized result of necrosis? The fact that transaminase and xanthine oxidase activities, which are also dependent upon coenzymes (pyridoxal phosphate and flavin adeninedinucleotide) are well maintained, and the fact of the low coenzyme A content of non-necrotic deficient liver tissue suggest that the coenzyme depletion of necrotic liver tissue is not nonspecific. It will be important, however, to make direct determination of other coenzymes in the livers of deficient rats, and these studies are in progress.

Is β-mercaptoethylamine, the sulfur moiety of coenzyme A, derived from dietary cystine? One can visualize a reasonably simple pathway from cystine to β-mercaptoethylamine via one step of reduction and one step of decarboxylation, although the order of the steps and all of the enzymes involved are not known. Studies with S^{35}-labeled cystine are in progress to verify this relationship and a search for the probable enzymes involved is under way.

Finally, the possible role in hepatic necrosis of another sulfur-containing coenzyme in the metabolism of pyruvate, α-lipoic acid must be ruled out.[16] Although this coenzyme is normally synthesized by mammalian tissues, it is conceivable that rigid organic sulfur restriction in the diet may also limit its synthesis.

The conclusions which can be drawn from this preliminary study are necessarily subject to qualification. I believe it can be said, however, that rat liver rendered necrotic by dietary restriction of cystine, methionine, and vitamin E presents an enzyme profile which features a defect in the oxidation of pyruvate which does not involve all enzymes of the citric acid cycle. It may be said, furthermore, that dietary restriction of the sulfur amino acids results in a lowering of coenzyme A equivalent to that seen in marked pantothenic acid deficiency prior to the onset of hepatic necrosis and suggests that a nonvitamin precursor of a tissue coenzyme may be as important in the elaboration of the coenzyme as the vitamin itself. Vitamin E protects against hepatic necrosis, tends to preserve the coenzyme A level, and appears to divert dietary methionine into channels other than the synthesis of xanthine oxidase.

Acknowledgments

It is a pleasure to acknowledge the technical assistance in these experiments of Suzanne Maury, Jean Sturm, Marcia Riegl, Ruth Neatrour, and Richard Shaw. The authors are also indebted to Dr. Frank Sherman of the Department of Pathology of the University of Pittsburgh School of Medicine for making the pathological observations.

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Discussion of the Paper

Doctor Klaus Schwarz: The results of Doctor Olson and his collaborators are in harmony with some of our own. Investigations in our laboratory have shown that the site of the primary metabolic defect in the dietary necrotic liver degeneration must be closely related to or identical with reactions of the citric acid cycle.[1, 2] Doctor Olson mentioned the hypothesis that the very low levels of coenzyme A in degenerating livers may be due to a defect in the utilization of cystine (or cysteine) for the formation of the thioethanolamine moiety of the coenzyme A molecule. We have accumulated some evidence which would tend to speak against this possibility. Thioethanolamine and also pantethine (*Lactobacillus bulgaricus* factor) were repeatedly tested for protective activity against dietary necrotic liver degeneration. Both substances were without effect under our experimental conditions.

References to the Discussion

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THE XANTHINE OXIDASE FACTOR (MOLYBDENUM)*

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The effect of diet on the xanthine oxidase activities of various rat tissues was studied as a sequel to an earlier investigation in which it was observed that dogs maintained on a purified diet developed a defect in acetaldehyde metabolism.[1] This defect was apparently due to the absence of some unidentified dietary factor involved in acetaldehyde metabolism. Of the known enzyme systems that could theoretically be concerned with acetaldehyde metabolism, the chemically similar xanthine and aldehyde oxidases seemed most likely to be dependent upon a dietary factor not already incorporated in the purified rations used. Since methods for studying xanthine oxidase were available, our attention was directed to a study of the relationship between tissue xanthine oxidase in rats and the dietary factors supplied to the animals.

Xanthine oxidase in rat tissues. At the time this work was started, riboflavin[2] and protein deficiencies[3] had been reported to decrease the concentration of liver xanthine oxidase in the rat. Our early studies were directed toward determining (1) the distribution of xanthine oxidase in rat tissues and (2) the tissues in which the xanthine oxidase concentration could be influenced by diet. The normal distribution of this enzyme was determined in adult rats maintained on a chow diet. The dietary studies utilized a purified ration identical or similar to that shown in TABLE 1. Low protein diets were prepared by replacing all or part of the casein with additional glucose.

The results of these studies[4] with adult rats are shown in TABLE 2. Small amounts of the enzyme were found in lung, kidney, spleen, and skin, but only the liver and small intestine contained large amounts. These two tissues were also the only ones in which the xanthine oxidase was markedly depleted by a purified low protein diet, and were the only tissues in which variations in xanthine oxidase could be expected from dietary deficiencies. The maximum effect of a protein deficiency was produced in two to four weeks, and no greater effect was obtained after six months. Considering the weight of each tissue, as well as its xanthine oxidase concentration, approximately half of the total amount of enzyme was present in the liver while 20 per cent was located in the skin and small intestine. Feeding a low protein diet removed at least 70 per cent of the total enzyme from the rat, but the amount remaining was adequate to maintain a normal excretion of uric acid and allantoin.[5] This deserves some emphasis because it indicates that either (1) uric acid can be formed by a mechanism not involving xanthine oxidase or (2) the amount of xanthine oxidase normally present in the rat far exceeds the amount required to fulfill its usual function. *In vitro* studies[6] could not detect any metabolic pathway for the formation of uric acid other than the one utilizing xanthine oxidase. The remaining conclusion is that rat tissues normally have at least a three-fold

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* The studies reported herein were supported by grants from the Nutrition Foundation, the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and the Office of Naval Research.

TABLE 1

COMPOSITION OF THE PURIFIED DIET USED IN STUDIES OF THE XANTHINE OXIDASE FACTOR

	gm.		mgm.
Casein (Labco)	24	Choline chloride	100
Crisco	4	Nicotinic acid	2.5
Wessen oil	2	Calcium pantothenate	1.0
Cod liver oil	1	Riboflavin	0.4
Salts (Phillips and Hart)*	4	Thiamine	0.4
Glucose	65	Pyridoxine	0.4

* P & H salts contain: Na, K, Ca, Mg, Fe, Mn, Zn, Cu, Cl, I, phosphate, sulfate, carbonate, and citrate.

TABLE 2

THE EFFECT OF FEEDING A PURIFIED 8 PER CENT CASEIN DIET ON THE XANTHINE OXIDASE ACTIVITIES OF ADULT RAT TISSUES

	Liver	Sm. Int.	Kidney	Lung	Spleen	Skin
Normal rats (on chow)	41	33	4	9	12	4
8% casein diet (4–28 weeks)	2*	6*	5	8	15	—
Normal (%) distribution	55	20	1	1	2	21

XO activities recorded as c.mm. O_2 per 20 minutes per 283 mgm. fresh tissue. At least 70 per cent of XO in entire rat is lost on a low-protein diet.

* The usual determination gives values of 0 and 2 for liver and intestine respectively. The recorded values represent the "true" amount present in relation to the normal values (see section on determination of xanthine oxidase).

reserve of this enzyme. Variations in tissue enzyme concentration or activity cannot automatically be interpreted as a corresponding variation in function.

Liver and intestinal xanthine oxidase. The weanling rat responded to low protein and other dietary changes more rapidly than the adult rat but otherwise similarly.[7] At weaning, rat liver contained only one third to one half of the adult level of xanthine oxidase. This could be increased to the normal adult level in about two weeks, when the rats were fed a complete natural diet of chow or a synthetic diet containing liver or milk. A purified synthetic diet containing adequate protein and riboflavin allowed slow increases to intermediate levels, but did not permit the attainment of normal values. The incorporation of liver in a diet containing inadequate protein did not modify the protein-deficiency depletion of liver xanthine oxidase. When the livers were first depleted of xanthine oxidase by a low-protein diet, the resumption of an adequate protein intake also restored the liver xanthine oxidase to about 75 per cent of the normal level.

It was concluded that the liver xanthine oxidase concentration depended primarily upon an adequate protein intake, but that the levels achieved with a purified diet were only 75 per cent or so of the values obtained with a diet containing natural foodstuffs such as liver or milk. It was postulated[7, 8] that liver, milk, *etc.*, contained some factor responsible for this effect. This factor could not be identified with any known nutritional substance, and was referred to as the liver residue factor or the xanthine oxidase factor (XOF). The magnitude of the effect of the xanthine oxidase factor on the concentration of liver xanthine oxidase was not large, but it was detectable and measurable.

Numerous successful tests were conducted, using the liver xanthine oxidase response as the criterion for the presence of this unidentified factor in the diet, but it was a difficult test to use as a routine assay procedure.

The source of XOF for the 75 per cent restoration of liver xanthine oxidase on a purified diet was unknown. In retrospect, it may have been derived from the bones. The relatively small effect of an XOF deficiency on liver XO could also be due to the availability of bone molybdenum for this response. The "dominant" effect of protein in controlling the level of liver xanthine oxidase may be an artifact because it might not be possible to produce a severe deficiency of molybdenum in the liver so long as preformed deposits exist in bones.

The effect of protein deficiency on other selected liver constituents has also been determined. The estrogen inactivating mechanism was lost,[9] and liver esterase,[10] glutathione,[11] riboflavin,[12] and choline oxidase[13] were severely depleted by protein deficiency. None of these was affected to the degree observed for xanthine oxidase.

Intestinal xanthine oxidase was markedly depleted by a purified diet containing adequate protein,[14] and this tissue was studied further as a possible test object for the xanthine oxidase factor. Rat intestine was "saturated" with xanthine oxidase at weaning by virtue of its previous milk diet. Feeding a purified diet which was deficient in either protein or the xanthine oxidase factor produced a very rapid depletion of xanthine oxidase from this tissue. A diet which was adequate in both protein and XOF maintained the high starting levels or rapidly restored high levels of intestinal XO. Both protein and XOF were required simultaneously for this response, and protein was not a dominant factor in the intestinal response as it was for liver XO.[15] With adequate protein in the diet, intestinal xanthine oxidase reflected the XOF content of the diet, and a bioassay procedure for XOF was established on this basis.[16]

Determination of xanthine oxidase. Since all of these results depended upon the determination of xanthine oxidase in rat tissues, the accuracy of the method used in determining this enzyme was studied. The results in this and other papers were obtained by the manometric procedure originally described by Axelrod and Elvehjem.[2] This method has been shown to give approximately correct values for liver when the latter contains a reasonable amount of enzyme, but does not measure correctly small amounts of enzyme in liver.[17] Nor does the method give correct comparative values for intestinal XO. Appreciable amounts of enzyme may be present in the intestine when this determination indicates a zero value.[18] A more satisfactory comparison could be made by determining the activity in the presence of methylene blue; or the activity could be determined in the usual manner and comparative relationships then calculated from the known and constant methylene blue relationship.[10, 18] In contrast to an homogenate, xanthine oxidase appeared to be relatively inert in a slice.[19]

FIGURE 1 shows the relative effects of protein and XOF deficiencies on liver and intestinal xanthine oxidases. The columns marked "detn." show the results obtained by the usual procedure for determining xanthine oxidase. The columns labeled "true" are the more correct relationships calculated from the values obtained in the presence of methylene blue. The true relationship shows

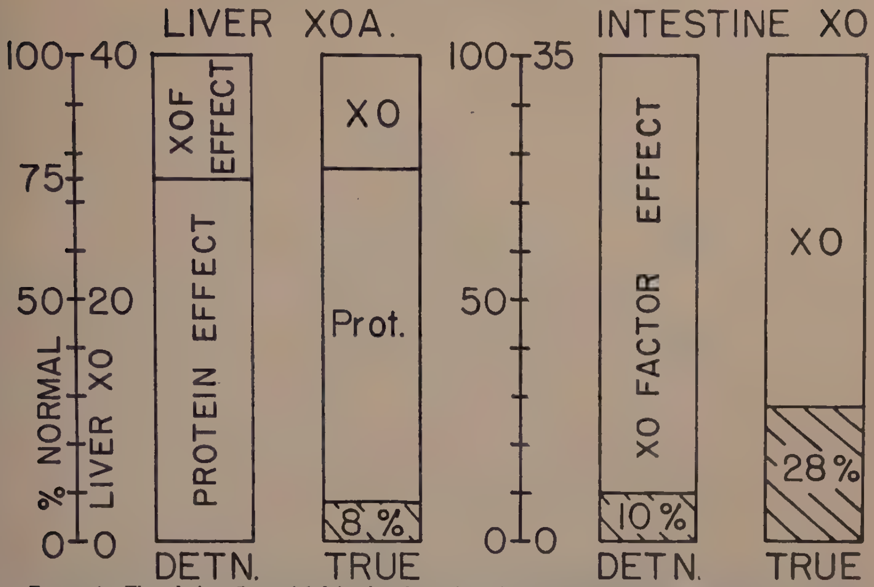

FIGURE 1. The relative effects of deficiencies of protein and xanthine oxidase factor on liver and intestinal xanthine oxidases. The XOF effect is obtained in the presence of adequate protein. Normal values for liver and intestinal XO were chosen as 40 and 35 (c.mm. O_2/20 min./283 mgm. fresh tissue) respectively. Cross-hatched areas are residual enzyme not removed by dietary deficiencies.

the presence of residual enzyme which cannot be removed by dietary means. The magnitude of the XOF effect (in the presence of adequate protein) is obviously much greater for intestine than liver.

Xanthine oxidase is not the same enzyme in all species. The enzyme in rat liver could be shown to have separate dehydrogenase and oxidase activities by the use of antabuse as an inhibitor of the oxidase portion of the enzyme.[20] The enzyme present in bird tissues was identified as a dehydrogenase rather than an oxidase,[21, 22] and the effect of dietary variations was shown[23] to be somewhat different for the dehydrogenase in bird tissues as compared with the oxidase in mammalian tissues.

Bioassay for XOF. Experience has shown that the greatest potential difficulty in the assay procedure previously described[16] is contamination of the casein used in the purified diet. In our early studies, control rats fed purified diets containing 31 per cent GBI vitamin-test casein had intestinal XO values averaging about 6 (c.mm. O_2 per 20 min. per flask containing 283 mgm. fresh tissue), and the published[16] assay curve proved very satisfactory. Later, however, the control levels of intestinal XO increased to 12 or 15 and occasional groups reached 20. Assay curves with such different batches of GBI casein are shown in FIGURE 2. The narrowing of the assay range and the increasing difficulty of the assay procedure as the control level of intestinal XO increased is evident. Since it is known that the hot alcohol extraction used for the purification of GBI casein does not remove XOF, the differences recorded in FIGURE 2 are dependent upon chance variation in the purity of the original untreated casein.

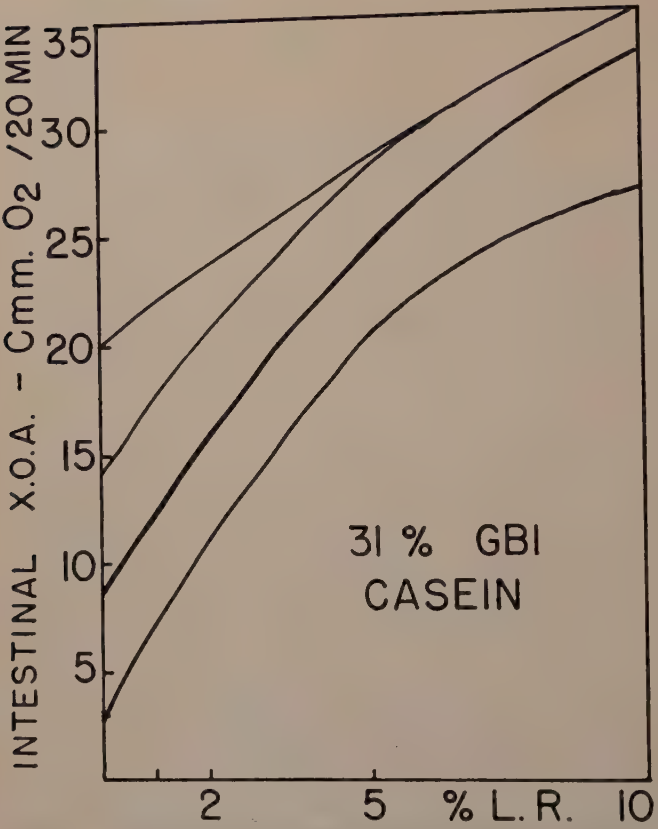

FIGURE 2. Various assay curves obtained with different batches of GBI casein.

A comparison of GBI casein with an acid-washed casein, Labco (Borden Co.), showed the latter to be far superior. Control intestinal XO values were 14 and 2 respectively for the two caseins. A control diet containing 24 per cent Labco casein has consistently given intestinal XO values of less than 3, and the bioassay curve obtained with this casein is shown in FIGURE 3. The upper curve shows the relationship obtained when the intestinal XO was determined in the presence of methylene blue. On this basis, dietary liver residue increased the intestinal XO threefold instead of the tenfold change indicated by the lower curve.

Diets with 24 and 31 per cent casein yielded similar or identical assay curves. The 24 per cent level was high enough to give a maximal protein response, and additional protein was without effect. A 21 per cent casein diet also yielded a similar assay curve, but the results seemed to be more erratic. The 24 per cent Labco casein diet was therefore adopted for routine testing.

Xanthine oxidase is apt to be localized in the mucosa of the intestine since muscles are generally free of this enzyme. To avoid possible removal of exposed mucosa by blotting the slit and washed intestine, the contents were ex-

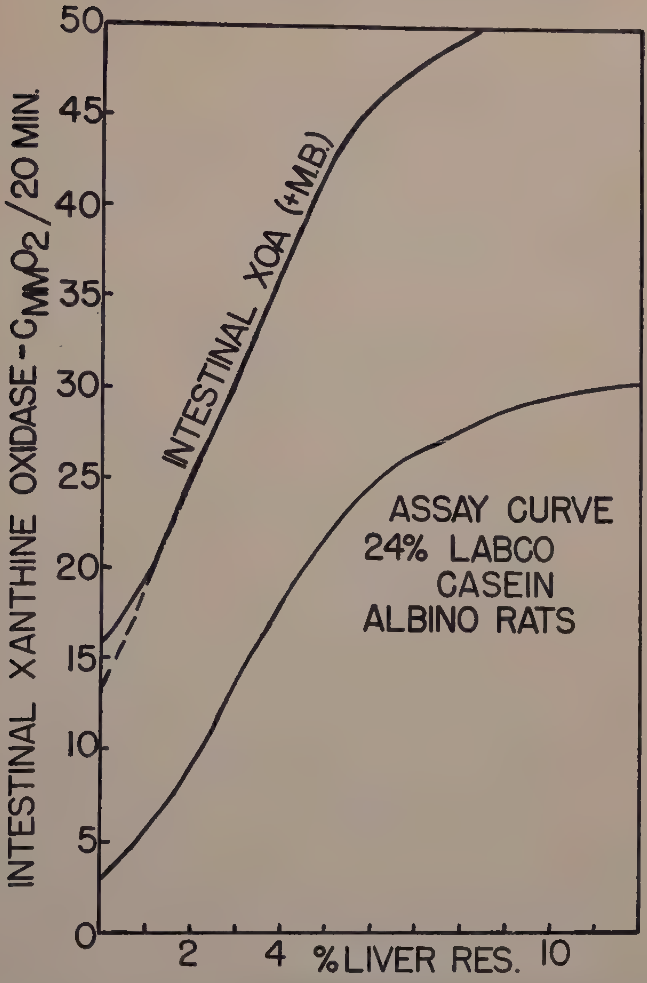

FIGURE 3. Assay curve for 24 per cent Labco casein diets containing varying amounts of liver residue. Upper curve is the same except that the intestinal xanthine oxidase values were measured in the presence of methylene blue.

pressed manually without exposing the mucosa. Such preparations were generally found to contain up to 25 per cent more XO than when the intestine was washed and blotted. Incompletely expressed or residual intestinal contents might theoretically contribute to the XO activity of the intestine, but there is little unexpressed material and the diet does not contain active xanthine oxidase.

Various foods have been assayed for XOF by incorporating the food in the diet being fed to weanling rats and measuring the xanthine oxidase activity of the intestine. Foods considered to be good or excellent sources of XOF gave an intestinal XO response equal to a 5 or a 10 per cent liver residue diet, and included (1) all cereals tested (except corn); (2) legumes; (3) kidney and liver;

TABLE 3

ISOLATION PROCEDURE FOR XOF

(1) Soy flour: reflux with 10 vol. of 1N HCl and filter.
(2) Filtrate: adsorb activity on charcoal and eluate with ammonia.
(3) Eluate: acidify, adsorb on alumina column, and elute fractionally with 1N ammonia.
(4) Eluate: adsorb on anion exchange resin (amberlite IRA-410). Wash with 0.2 N HCl. Elute alternately with 1N NaOH and HCl.
(5) Eluates: acidify, adsorb on charcoal, and elute with ammonia.
(6) Eluate: concentrate, precipitate with $BaCl_2$ and alcohol.
(7) Ba Salt ppt.: fractional crystallization from water.

and (4) some dark green, leafy vegetables. Foods which were essentially inactive included (1) muscle meats and fish, and (2) fruits and berries. The substance present in liver residue and foods, which was responsible for this response could not be identified with any factor known to be required in animal nutrition. Nor could it be identified with the antithyrotoxic factor present in liver residue.[24]

Isolation and identification of XOF. The material present in soy flour giving this response was isolated by a series of fractionation procedures in which the active material was followed throughout its purification by bioassay. The major steps of the isolation procedure are shown in TABLE 3. Two active barium salts were separated from the final barium precipitate. One was insoluble in water, lost little weight on ashing, and contained 45 per cent barium and 24 per cent molybdenum. Spectrographically, it contained only Ba and Mo in amounts greater than 0.1 per cent. The other barium salt could be crystallized from hot water, lost a significant proportion of its weight on ashing, and contained about 17 per cent molybdenum. Although the purification procedure was not designed to yield inorganic material specifically, nevertheless the isolated substances were molybdate or molybdate-complex salts.

The identification of these substances as molybdates was aided by the concurrent studies of De Renzo *et al.*[25] Using the same assay procedure, they found that the biological activity was retained in a liver residue ash, and they identified molybdenum as the active component of the ash by testing each of its constituents.

The biological activity of sodium molybdate is shown in FIGURE 4. Saturation levels of intestinal xanthine oxidase (above 30) were obtained with diets containing less than 0.1 mgm. molybdenum per kgm. of diet. Higher concentrations of dietary molybdenum gave intestinal XO values averaging between 30 and 45, but they were not consistently proportional to the molybdenum content of the diet. Hence values above 30 were considered to be saturation levels of intestinal XO, and assays falling within this range were repeated at a lower level.

Having established molybdenum as a major component of the active material isolated from soy flour, it remained to be determined whether all of the biological activity of this substance could be accounted for by its molybdenum content. The following evidence demonstrated conclusively that such was the case:

(1) The activity of the isolated material or of a large number of impure frac-

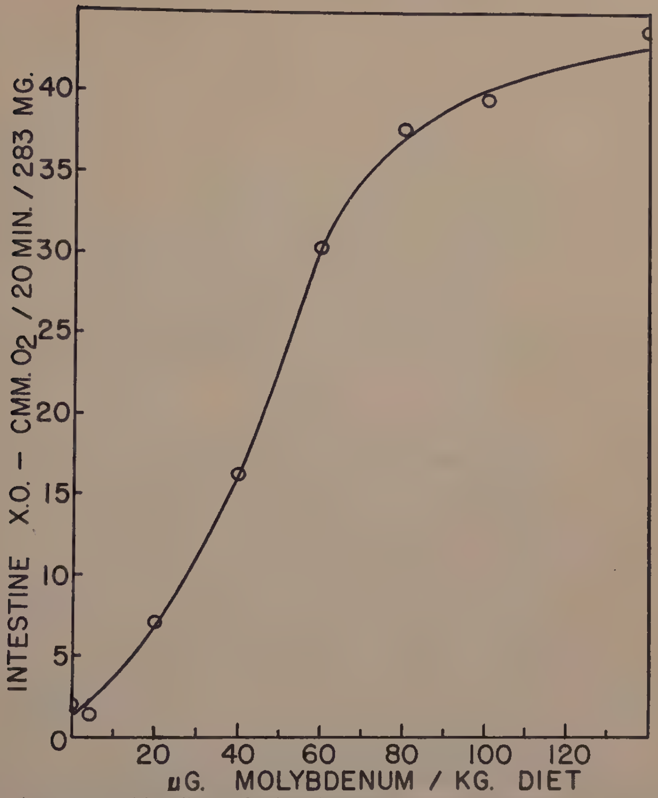

FIGURE 4. Assay curve with sodium molybdate showing the response of intestinal xanthine oxidase to increasing amounts of molybdenum in the diet.

tions was identical with that of pure sodium molybdate when tested on the basis of their respective molybdenum contents.

(2) The molybdenum content and the biological activity of the isolated material were not decreased by ashing. Hence no essential or synergistic organic component was carried along with the molybdenum during the isolation procedure.

(3) A removal of molybdenum from the isolated material by extracting it as an ether soluble thiocyanate complex also removed the biological activity with the molybdenum.

(4) Precipitation of molybdenum from the isolated material as an insoluble comples with α-benzoinoxime also precipitated the biological activity.

(5) Liver residue and soy flour contained more than enough molybdenum to account for their biological activities.

These results leave no doubt that the xanthine oxidase factor is the trace element molybdenum. Molybdenum is known to be required by some plants and microorganisms, but this is the first demonstration of a role for molybdenum in animal nutrition and is the first association of molybdenum with an enzyme.

It appears to be a part of the enzyme molecule, since purified preparations of xanthine oxidase contain molybdenum. This was demonstrated previously[16] by the assay procedure, and has been confirmed more recently by chemical determinations.

Studies of molybdenum deficiency in plants[26, 28] and *Aspergillus niger*[29] have demonstrated a requirement for molybdenum in the reduction of nitrate. Xanthine oxidase[30] and aldehyde oxidase[31] are capable of utilizing nitrate or organic nitro compounds[32, 33] as electron acceptors during the oxidation of purine or aldehyde substrates. Nitrate is thereby reduced to nitrite,[30] and organic nitro compounds are reduced to hydroxylamino derivatives.[33] It seems probable, therefore, that the reduction of nitrates by plants, molds, *etc.*, is effected by a molybdenum-containing enzyme similar to but not necessarily identical with xanthine oxidase.

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STUDIES ON THE NATURE OF THE XANTHINE OXIDASE FACTOR*

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I should like to extend my thanks to Doctor Schwarz and to Doctor Westerfeld for the opportunity of presenting a brief summary of some of our findings on the nature of the xanthine oxidase factor. I should like to extend special thanks to Doctor Westerfeld for his gracious acknowledgment of our contribution.

When we first became interested in this dietary factor, a study of the literature revealed that the Syracuse workers were practically alone in thinking about a substance other than adequate protein or riboflavin which was concerned with maintaining tissue xanthine oxidase values. In order to satisfy ourselves that such a factor actually existed, we attempted to reproduce the experiments of the Syracuse group. Preliminary experiments satisfied us that the feeding of liver residue to weanling rats results in an increased xanthine oxidase level in the small intestine. Difficulty was experienced, however, in obtaining a linear response in the intestinal xanthine oxidase value to varying amounts of dietary liver residue and, in addition, rats receiving the control diet alone showed relatively high levels of this enzyme. After a brief talk with Doctors Westerfeld and Richert, our problems with the assay were satisfactorily unraveled.

TABLE 1 shows the basal diet[1] used in these studies. As reported by the Syracuse workers, if weanling rats are given this diet for two weeks, analysis of the small intestine for xanthine oxidase by the manometric technique[2] will yield a value of about 5 c.mm. oxygen uptake/20 min./250 mgm. intestine. Supplementation of this diet with 10 per cent liver residue produces a five- to sevenfold increase in the xanthine oxidase value. TABLE 2 shows the response of rat intestinal xanthine oxidase to varying amounts of liver residue in the diet thus confirming the work already reported.[1] It should be mentioned, however, that we sometimes experienced difficulty in obtaining xanthine oxidase values proportional to the lower amounts (*i.e.*, 2–5 per cent) of a liver residue supplement. Nevertheless, there was never any doubt about the enhancement obtained with a high level (*i.e.*, 10 per cent) of liver residue, and isolation studies were therefore undertaken.

We chose liver residue as our source material since the factor was present in abundant amounts in this material. Early in the work, it was found that active extracts of xanthine oxidase factor could be obtained by simply autoclaving liver residue with water. The factor in the extract as well as in the crude liver residue was remarkably stable to acid or alkaline treatment and, when the activity was not destroyed by boiling with concentrated sulfuric acid, our suspicions as to the nature of the factor were aroused. We therefore ashed the active extracts and liver residue; and when the ashed materials were fed, no diminution of activity was encountered. These data are shown in TABLE 3.

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* The capable assistance of Mr. E. Kaleita and Mr. P. G. Heytler in the performance of these experiments is gratefully acknowledged.

TABLE 1

BASAL DIET USED IN STUDIES ON XOF

Cerelose	65
Purified casein (Labco)	24
Crisco	4
Cod liver oil	1
Wesson oil	2
Salts (Phillips and Hart[4])	4

Vitamins/kgm. diet.
Riboflavin, 4 mgm.
Thiamin·HCl, 4 mgm.
Pyridoxine·HCl, 4 mgm.
Ca Pantothenate, 10 mgm.
Nicotinamide, 25 mgm.
Choline chloride, 1000 mgm.

TABLE 2

RESPONSE OF RAT INTESTINAL XANTHINE OXIDASE VALUES TO LIVER RESIDUE IN THE DIET

Amount of liver residue added to basal diet (per cent)	Av. X. O. value c.mm. O_2 uptake/20 min./250 mgm. intestine
None	5.0
2	10.2
5	22.0
10	29.8

TABLE 3

EFFECT OF LIVER RESIDUE FRACTIONS ON RAT INTESTINAL XANTHINE OXIDASE VALUES

Supplement added to basal diet	Av. X. O. value c.mm. O_2 uptake/20 min./250 mgm. intestine
None	4.4
10% liver residue (LR)	25.6
Liver residue water extract (LRE)	32
Ash of LR	28.2
Ash of LRE	26.9

A spectrographic analysis (TABLE 4) of the ash of the active extract revealed the presence of many elements. The activity of the ash was then found to be replaceable by Hoagland's A–Z solution (50 ml./kgm. diet), an inorganic solution containing trace elements and used in studies of plant nutrition.[3] Single salts were then fed in varying amounts in an attempt to ascertain the active substance. On the basis of the elemental composition of the ash and Hoagland's solution and the salt mixture of the basal diet, our studies were restricted to relatively few salts. To our surprise, molybdenum salts were found to be responsible for this effect. These data are shown in TABLE 5. No other salts studied exhibited this effect, and the activity of the ash of active extracts or liver residue could be totally accounted for on the basis of its molybdenum content. Furthermore, and also to our surprise, when an ash of the basal diet was assayed spectrographically, molybdenum was not detected (TABLE 6).

We have attempted to study the effects of molybdenum on milk as well as

TABLE 4
SPECTROGRAPHIC ANALYSIS OF LIVER RESIDUE ASH

Element detected	Approximate per cent (unless Stated as parts per million, ppm.)
Al	0.3
Sb	Trace
Ba	0.1
B	1
Ca	1
Cr	30 ppm.
Co	0.1 ppm.
Cu	0.1
Fe	1
Pb	0.3
Mg	10
Mn	30 ppm.
Mo	0.3
Ni	30 ppm.
P	3
K	3
Si	10
Ag	30 ppm.
Na	30
Sn	0.03
Ti	10 ppm.
V	0.03
Zn	0.1

TABLE 5
EFFECT OF CERTAIN SALTS ON RAT INTESTINAL XANTHINE OXIDASE VALUES

Supplement added/kgm. diet	Av. X. O. value c.mm. O_2 uptake/20 min./250 mgm. intestine
None	7.1
50 ml. Hoagland's solution	21.4
$AlCl_3 \cdot 6H_2O$, 200 mgm	5.3
$CoCl_2 \cdot 6H_2O$, 200 mgm	1.1
$Cr(NO_3)_3 \cdot 9H_2O$, 200 mgm	3.7
$SnCl_4 \cdot 5H_2O$, 200 mgm	3.4
$SrCl_2 \cdot 6H_2O$, 200 mgm	3.7
H_3BO_4, 100 mgm	8.6
NaBr, 25 mgm	4.9
$K_2Cr_2O_7$, 25 mgm	4.8
$Ti(OH)_4$, 25 mgm	6.7
$Ni(NO_3)_2 \cdot 6H_2O$, 10 to 100 mgm	9.5
$BaCl_2$, 10 to 50 mgm	3.7
$Na_2MoO_4 \cdot 2H_2O$, 0.05 to 100 mgm	27.3

TABLE 6
ELEMENTS DETECTED IN THE BASAL DIET ASH SHOWING THE ABSENCE OF MOLYBDENUM

Al
Ba
Ca
Cr
Cu
Fe
Pb
Mg
Mn
Ni
P
K
Si
Ag
Na
Sn
Zn

intestinal xanthine oxidase *in vitro*. Varying concentrations of molybdate were added to dialyzed or undialyzed active enzyme preparations and enzyme preparations from rats receiving the basal diet (*i.e.*, deficient in molybdenum). No enhancement of activity has yet been observed under these conditions with any of these enzyme preparations.

In preliminary experiments, we have found that the feeding of sodium tungstate to rats receiving minimal amounts of molybdenum inhibits the stimulation of intestinal xanthine oxidase. This appears to be an interesting sort of ion antagonism. However, no inhibitory effect of tungstate on milk or intestinal xanthine oxidase preparations has been observed *in vitro*.

We have also demonstrated an *in vivo* stimulatory effect of molybdenum on mouse intestinal xanthine oxidase and therefore feel that the participation of Mo in animal nutrition and metabolism has been firmly established. Further attempts are now in progress to demonstrate the precise role of molybdenum on the activity of xanthine oxidase.

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THE EFFECTS OF LIVER DISEASE ON CERTAIN ASPECTS OF TOCOPHEROL METABOLISM IN MAN*

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The purpose of the present investigation was to characterize the effects of liver disease on certain aspects of tocopherol metabolism in man. Our interest in this problem was stimulated by the observation that chronic biliary fistula leads to vitamin E deficiency in experimental animals,[1, 2] an effect thought to be the consequence of impaired tocopherol absorption. Although clinical evidence of vitamin E deficiency has not been recognized in man, a similar defect in absorption has been postulated to account for the low levels of plasma tocopherol found in liver disease.[3, 4] The subnormal rise in the plasma level following the administration of tocopherol in such cases[3, 4] would appear to lend support to this hypothesis. It is obvious, however, that the plasma level reflects not only the rate of tocopherol absorption in the intestine, but also its rate of storage and utilization in the tissues. Unfortunately, little is known about these aspects of tocopherol metabolism in man, so that the significance of low plasma levels has remained obscure. However, recent evidence implicating tocopherol deficiency in the pathogenesis of experimental dietary hepatic necrosis[5, 6, 7] has served to emphasize the possible importance of this problem. The following studies were undertaken, therefore, with the hope of shedding further light on the interrelationships between hepatic dysfunction and the absorption, utilization, and storage of tocopherol in liver disease.

Results

Plasma tocopherol. Analyses of plasma confirmed previous reports[4] that the average tocopherol level in liver disease is significantly lower than in normal healthy adults (FIGURE 1). It was found, however, that the individual values varied widely, often falling within the normal range, and that the average did not differ significantly from that of a group of randomly-selected hospitalized convalescent controls. Moreover, there was no correlation between the concentration of tocopherol and the depth of jaundice or the degree of hepatic dysfunction, as judged from the serum levels of bilirubin, cholesterol, and albumin (FIGURE 2).

These observations suggested that the decrease in plasma tocopherol in liver disease was related to some extrahepatic factor, such as impaired absorption, rather than to a metabolic defect in the liver. However, a depletion of the tissues and plasma due to a dietary deficiency of tocopherol had to be excluded, since many of the subjects in both the liver disease and convalescent control groups had subsisted on grossly deficient diets for long periods.

Saturation tests. If the fall in plasma tocopherol were indeed related to such a deficiency, repletion of the tissues might be expected to effect a sustained rise in the plasma tocopherol level. For that reason, the effects of large amounts

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* This investigation was supported by a research grant (A-47(C)) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U. S. Public Health Service.

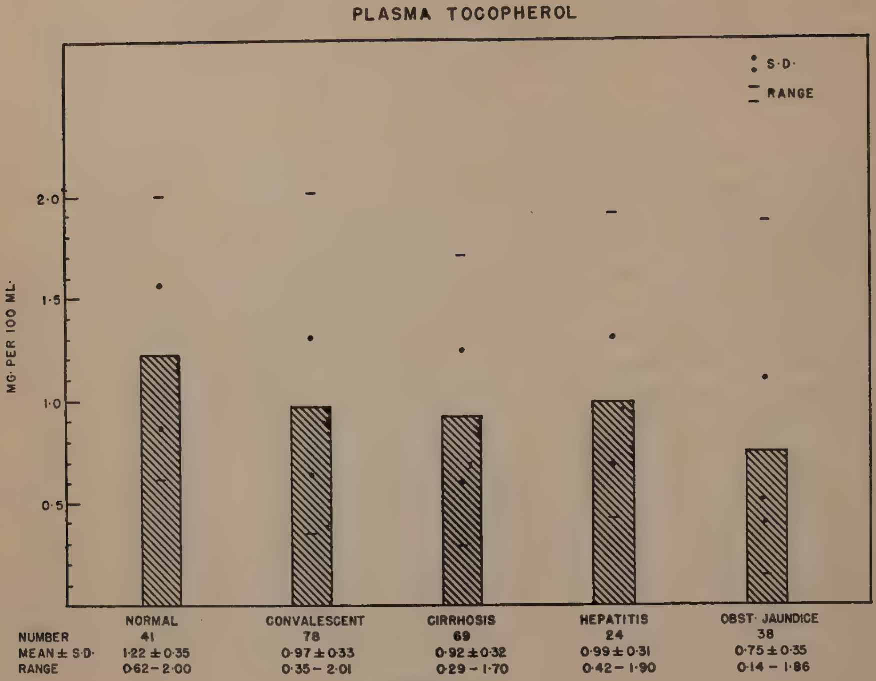

FIGURE 1

of orally administered tocopherol were investigated. (The 4 gm. dose of acetate employed approximated the estimated total body content of tocopherol.)[8]

It can be seen in A and B of FIGURE 3 that, with the exception of the obstructive jaundice group, the subjects with liver disease showed as great a rise in plasma concentration during tocopherol administration as the normals, suggesting that absorption from the intestine was not impaired. However, as is evident in D of FIGURE 3, the rise was sustained in the cirrhotic group only. In the latter, moreover, the absolute increase in concentration over the control level actually exceeded that in the normals (C of FIGURE 3).

These data suggested that dietary deficiency was at least one of the factors responsible for the low plasma tocopherol levels in cirrhosis. If it played any role in the other types of liver disease investigated, it was obscured by other more important factors.

Tolerance tests. To further evaluate the factor of intestinal absorption, tolerance tests based on plasma curves following the oral administration of tocopherol were carried out. These confirmed previous reports[4] that the curves in liver disease tended to be lower and flatter than normal (A of FIGURE 4). Further analysis, however, revealed that while the peak values attained were low (B of FIGURE 4), the increases above the initial fasting level (C of FIGURE 4), and the mean rises in concentration estimated from the areas under the curves (D of FIGURE 4), were within normal limits, except in the case of obstructive jaundice.

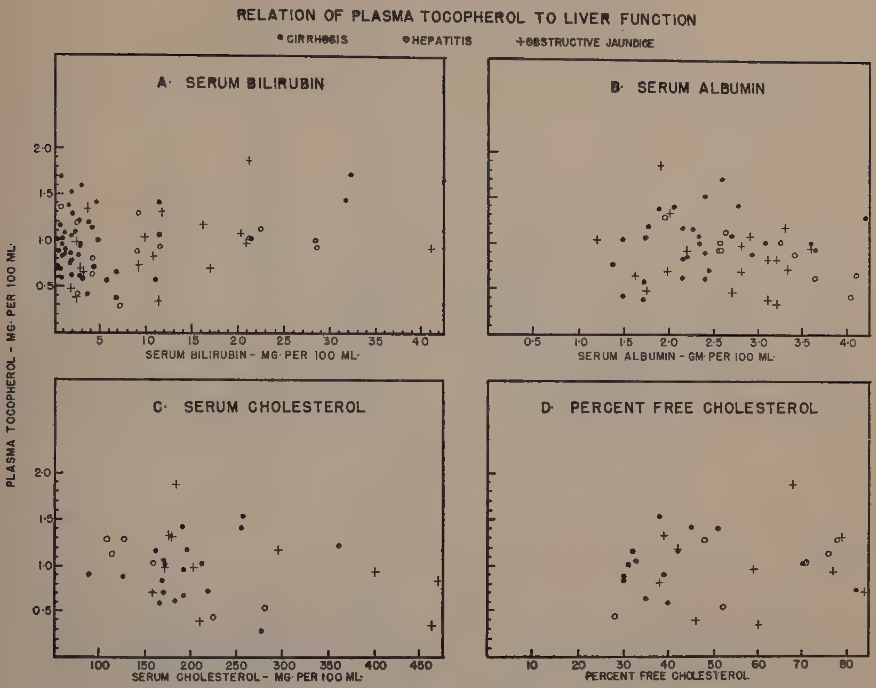

FIGURE 2

As in the saturation studies, the results suggested that there was no impairment of tocopherol absorption, at least in the cirrhotis and hepatitis groups. It was recognized, however, that estimates based on plasma curves did not warrant definite conclusions regarding intestinal absorption, unless paralleled by measurements of tocopherol excretion. For that reason, it was necessary to carry out a number of balance experiments. Since preliminary investigation revealed that little or no tocopherol was excreted in the urine, these were based on analyses of feces.[9]

Fecal tocopherol. To our surprise, it was found that subjects with liver disease regularly excreted a smaller fraction of ingested tocopherol than normals, both on their usual diets (A and B of FIGURE 5) and following the ingestion of a large tocopheryl acetate supplement (C of FIGURE 5).

Several possible explanations for the low fecal tocopherols in liver disease were considered:

(1) A larger fraction of ingested tocopherol was absorbed; (2) A fraction of the ingested tocopherol was excreted as the acetate and, therefore, escaped detection in the Emmerie-Engel reaction, which served as the basis for the analytical procedure employed;[10]

(3) There was interference with some as yet unrecognized normal mechanism for the re-excretion of tocopherol into the intestine;

(4) Synthesis of tocopherol occurred in the intestinal tract of the normals and was diminished in the subjects with liver disease;

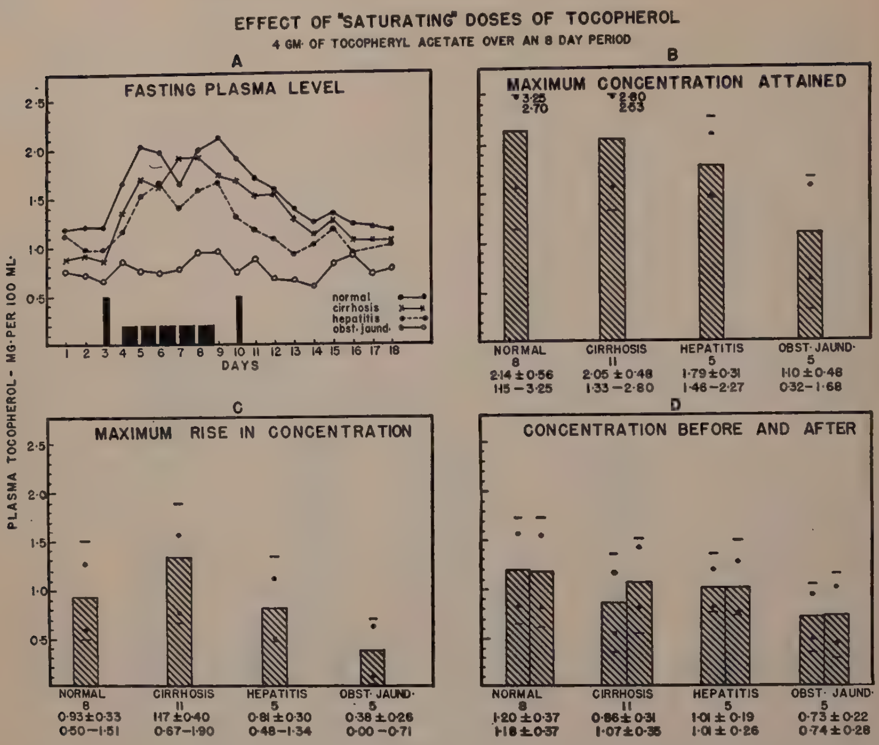

FIGURE 3

(5) There was an increased rate of tocopherol breakdown in the intestinal tract.

(*1*) *Increased absorption.* If the subjects with liver disease did in fact absorb a larger fraction of ingested tocopherol, it would have to be assumed that the relatively low or normal plasma curves observed during tocopherol absorption (FIGURE 4) were the consequence either of an increased rate of utilization or of a more rapid diversion of tocopherol to the tissues. Under these conditions, loading of the plasma and tissues with large amounts of tocopherol might be expected to result in a relative decrease in the rate of utilization and/or transfer to the tissues. Actually however, when tolerance tests were carried out before and immediately after saturating doses of tocopherol, the reverse was found to be the case. Although the peak levels attained after saturation were higher than before (A and B of FIGURE 6), the absolute increases above the fasting level (C of FIGURE 6) and the mean rises (D of FIGURE 6) were either lower or not significantly different. Thus, saturation of the tissues did not decrease the rate at which tocopherol was diverted to the tissues; nor, if the slope of the curves can be used as an index, did it alter the rate of tocopherol utilization. Unfortunately, more direct means for measuring utilization could not be devised. Nevertheless, it was concluded tentatively that the low fecal tocopherol levels in liver disease were not due to an increase in tocopherol absorption.

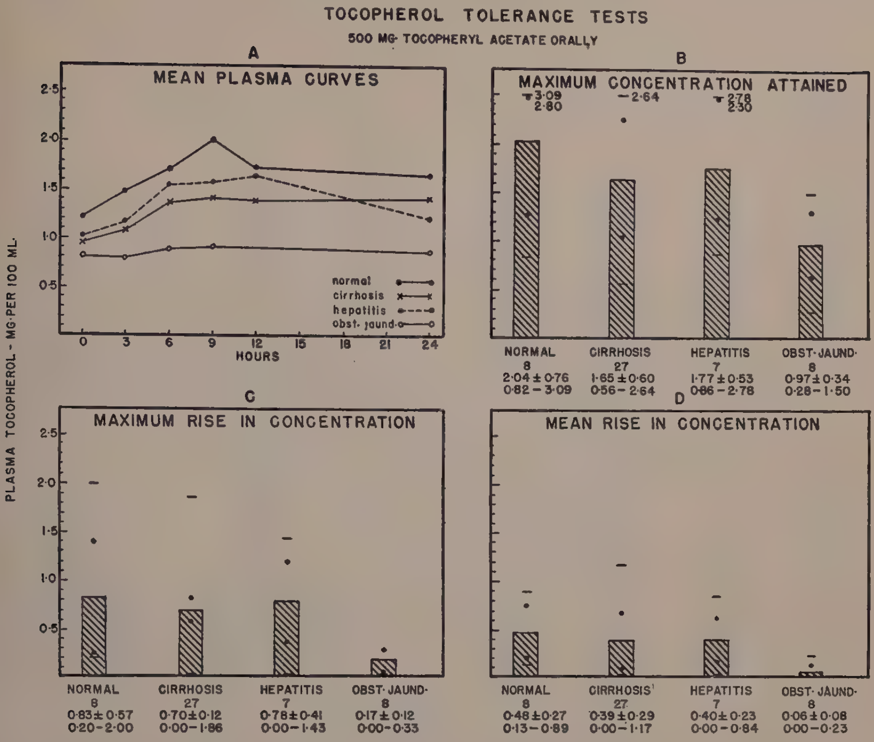

FIGURE 4

(2) *Tocopheryl esters.* When the feces were saponified to free tocopherol from its esters prior to analysis, it was found that some but not all of the subjects with liver disease were excreting small amounts of tocopheryl esters (C of FIGURE 7). The amounts were far too small, however, to account for low levels of fecal tocopherol on the basis of analytical errors due to the presence of esters. Thus, for example, it will be noted that the subject with the lowest excretion (C of FIGURE 7) had no esters whatever in his feces.

It was of interest, nevertheless, to investigate the nature of the disturbance in liver disease responsible for the appearance of tocopheryl esters in the feces. *In vitro* incubation studies revealed that the acetate was readily hydrolyzed by aspirated jejunal contents (A of FIGURE 7), but not by pure cultures of enteric organisms (B of FIGURE 7), indicating that the esterases responsible for this breakdown were derived from the intestinal secretions and/or bile. This was demonstrated more directly by measuring fecal tocopheryl esters following the instillation of tocopheryl acetate distal to an occlusive balloon in the jejunum and the continuous aspiration of the duodenal contents for a period of 30 hours. It can be seen in D of FIGURE 7 that large amounts of the ester were excreted in both normal subjects and cirrhotics under these conditions.

Further study is needed to identify the source of this esterase, and the reason for its reduction in liver disease.

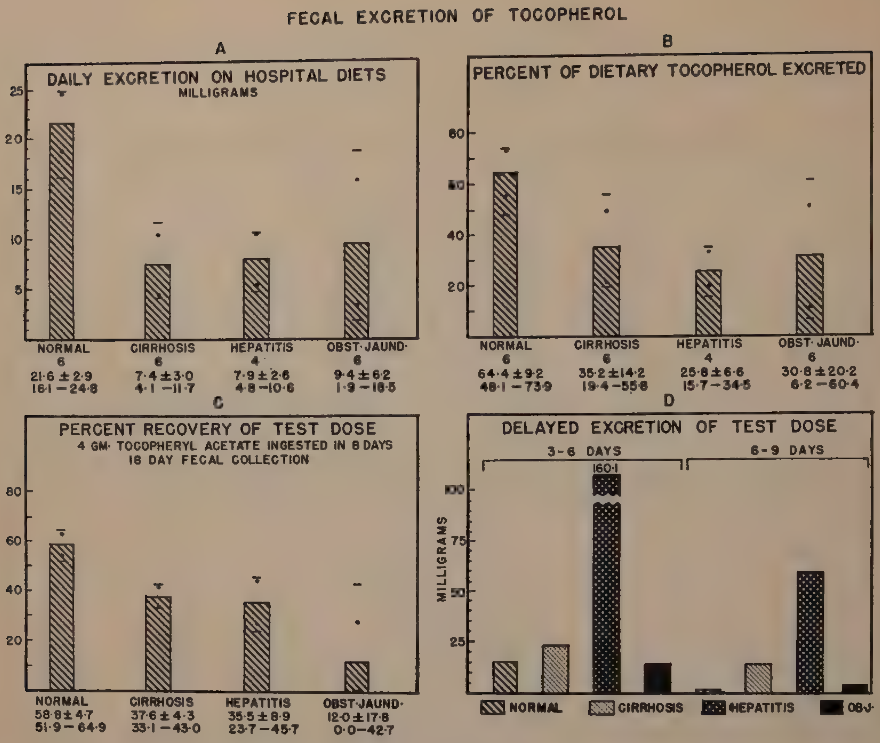

Figure 5

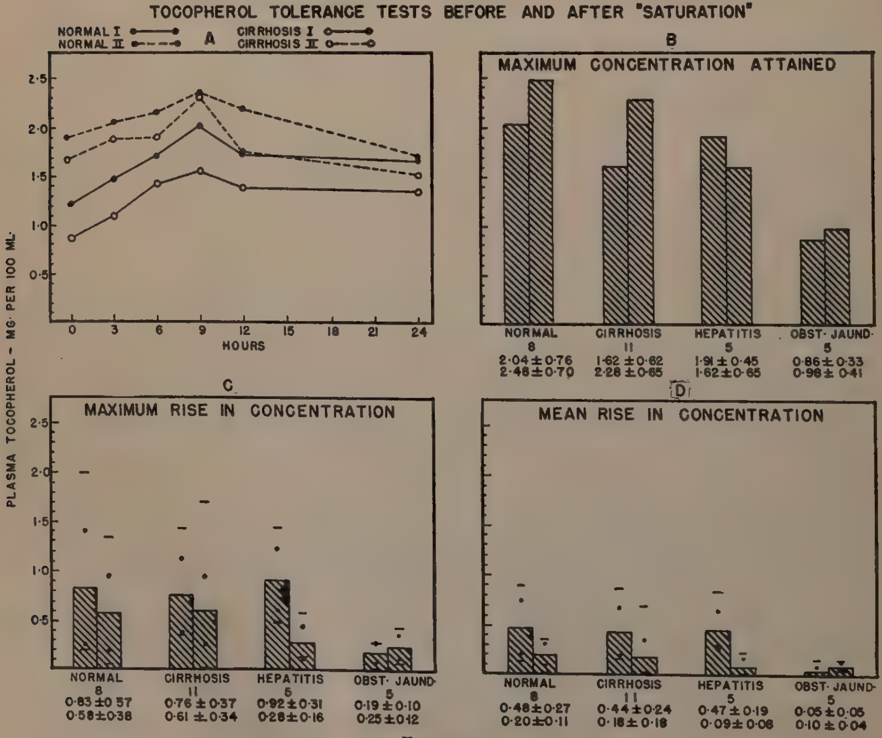

Figure 6

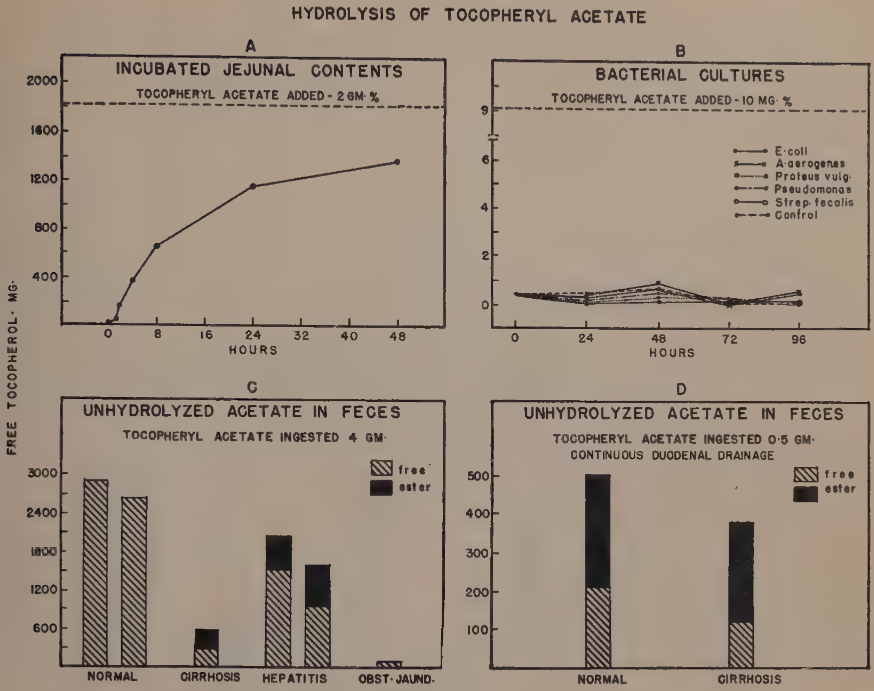

FIGURE 7

(3) *Re-excretion of tocopherol.* The possibility that there was interference with some normal mechanism for the re-excretion of tocopherol into the intestinal tract, which led to a reduction in fecal tocopherol, was suggested by the observation that the amount excreted in liver disease remained significantly above the baseline level for at least 9 days following an oral test dose of tocopherol (D of FIGURE 5). Partial confirmation of such a mechanism was obtained from analyses of T-tube bile in subjects with obstructive jaundice. These revealed concentrations of tocopherol comparable to those in plasma (A of FIGURE 8). However, it was not possible to demonstrate any interference with this enterohepatic circulation of tocopherol in liver disease. Thus, analyses of duodenal contents aspirated for a period of 24 hours following instillation of tocopherol distal to an occlusive balloon in the jejunum failed to reveal any difference in the amount recovered in normal and cirrhotic subjects (C of FIGURE 8). Moreover, the fact that the amount recovered was small, and the observation that the concentration in T-tube bile did not increase following the ingestion of tocopherol (B in FIGURE 8), suggested that the bile contributed very little to the total fecal tocopherol in the previously described balance experiments.

It was concluded, therefore, that interference with the enterohepatic circulation of tocopherol was not responsible for the low fecal levels found in liver disease. The possibility of a defect in its re-excretion, however, through the intestinal mucosa could not be excluded, although it appeared unlikely.

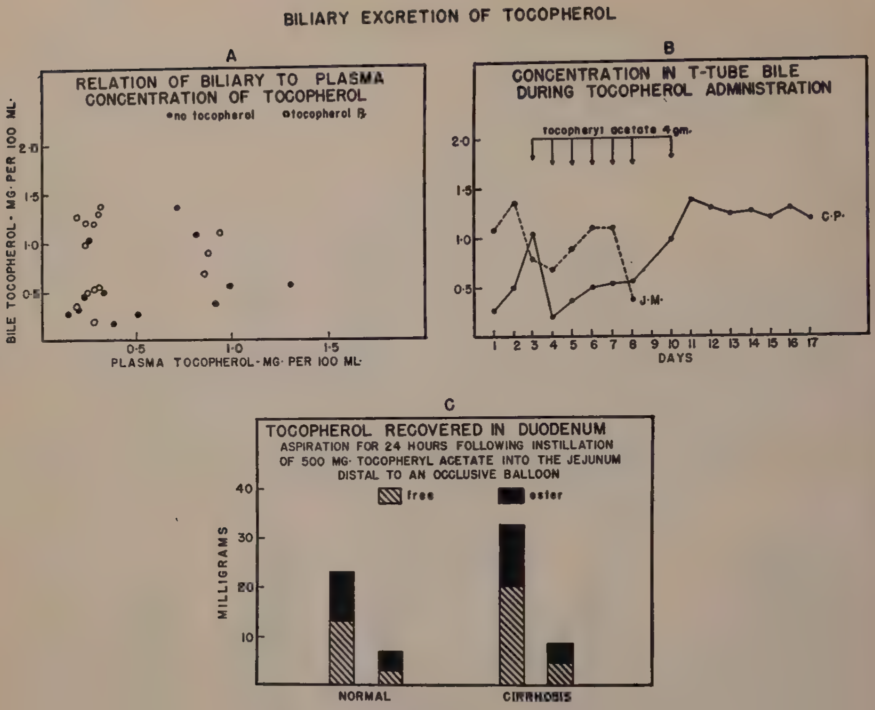

FIGURE 8

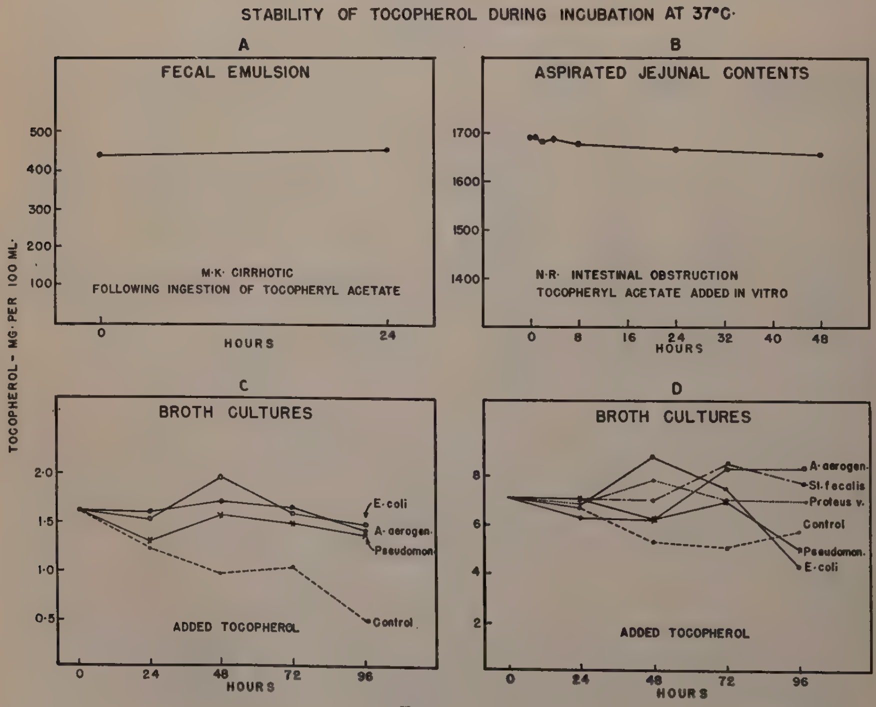

FIGURE 9

(4) *Tocopherol synthesis.* The possibility of tocopherol synthesis in the intestinal tract was investigated, although there was no previous evidence to indicate that it was a factor in determining the level of tocopherol in the feces. Attempts to demonstrate such synthesis in cultures of a broad spectrum of aerobic and anaerobic enteric organisms grown in a variety of enriched media, or in incubated emulsions of feces and aspirated intestinal contents, were uniformly unsuccessful.

(5) *Tocopherol "destruction."* Largely by exclusion, an increased rate of destruction in the intestinal tract appeared to be the most likely explanation for the low fecal tocopherol in liver disease. It was not possible, however, to demonstrate this by direct experiment. Thus, there was no loss of tocopherol during its prolonged incubation in fecal emulsions (A of FIGURE 9), aspirated jejunal contents (B of FIGURE 9), or cultures of enteric organisms (C and D of FIGURE 9). Nevertheless, it must be emphasized that these experimental conditions by no means duplicated those in the intestinal tract, so that differences in tocopherol destruction were not excluded. The recent observations of Rosenkrantz and his associates[11] are highly significant in this connection. These investigators found that an appreciable fraction of the tocopherol in normal feces was excreted as the quinone, a form not detectable by the Emmerie-Engel reaction. It is conceivable, therefore, that a more rapid oxidation of tocopherol to tocopherylquinone in the intestinal tract was responsible for the low fecal values obtained by the Emmerie-Engel reaction in the present experiments. Further studies along these lines are contemplated.

Summary

In summary, then, previous reports of low plasma tocopherol levels in liver disease have been confirmed. The evidence presented, however, suggests that these low levels are not the result of impaired absorption, but are, rather, the consequence of an increased rate of tocopherol breakdown in the intestinal tract. Moreover, in some instances dietary deficiency appears to be a contributory factor.

Acknowledgment

The author is indebted to Doctor David W. Molander, Doctor Willard A. Krehl, Doctor Daniel Marshall, and Miss Rita Pacelli for their assistance in carrying out these studies.

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PHOSPHOLIPIDE SYNTHESIS IN LIVER METABOLISM*

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Phospholipide synthesis is a primary function of all cells in the body. They make up part of the cell structure, being found in both the cytoplasma and nucleus. Mitochondria and large granules are very rich in phospholipides.[1] These intracellular structures contain enzymes which can oxidize fatty acids[2] and other metabolites. The liver is the main source of phospholipides in plasma.[3] Plasma phospholipides show a direct reflection of turnover in the liver.[4, 5] The choline-containing phospholipides are probably an integral part of enzyme systems necessary for the oxidation of fats and fatty acids. The lipotropic effect of lecithins appears to act on the metabolism of fatty acids in the liver, rather than by enhancing their mobilization in the form of plasma phospholipides. The composition of liver lipides can be altered by the dietary intake of proteins.[6, 7] There is usually a decrease in the level of total phospholipides in the liver of animals maintained on a low-protein diet. The decrease is especially marked in the lecithin fraction with a consequent lower ratio of the choline-containing to the total phospholipides. Various studies comparing the rate of incorporation of P^{32} into the liver lipides of animals on various experimental diets have been made.[6, 7, 8]

The phospholipide turnover in the plasma of normal individuals varies widely, but is rather constant in the same individual over a period of many months.[9] The effect of a low-protein diet (7.6 per cent) or a normal diet for 9 days seems to have very little change in the rate of synthesis of the plasma phospholipide in man.[10] The phospholipides of the plasma are primarily choline-containing; but in the liver, both choline and noncholine-containing are abundant.[11, 12] Attempts have been made to study the effects of dietary requirements on the synthesis of these two fractions in the liver.[13] The synthesis of the choline-containing fraction was practically the same in the liver of animals maintained on a low-protein diet (5 per cent) as in those on a stock diet or a 25 per cent casein diet. The only instance in which a marked decrease in synthesis of the choline-containing fraction were those of experiment, in which guanidioacetic acid or diethanolamine was supplemented.[14] Diethanolamine apparently acts as a metabolic antagonist of ethanolamine for the formation of lecithin as well as the natural cephalin.[15] The rate of lecithin synthesis by the liver of the rat is apparently related to the activity of choline oxidase, or to the role of natural inhibitors such as fatty acids.[16] This diminished choline oxidase activity permits an adequate rate of synthesis of phospholipides containing choline. It is impossible to produce a "fatty" liver by dietary means in the liver of the guinea pig, since this enzyme is not present.[17] The liver cell is more efficient with the lecithin molecule when the liver is "fatty," even though a decrease in the total phospholipide P occurs.

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* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service, and the North Dakota Cancer Society.

TABLE 1

PHOSPHOLIPIDE SYNTHESIS IN THE RAT AND GUINEA PIG ON STOCK DIET*

Animal	No.	Body weight gm.	Phospholipide-P			Phospholipide-P/Nitrogen	Relative radioactivity per gm. of fat free tissue
			mgm.	Relative specific activity			
				overall	per gm. of fat free tissue		
Rat	8	278	17.4	0.386	0.040	0.054	0.68
Guinea pig	6	989	33.5	0.258	0.009	0.042	0.30

* Animals killed 6 hours after the injection of P^{32}.

A comparison of phospholipide synthesis in the guinea pig and rat has been made in an attempt to determine the role of choline oxidase in lipide phosphorylation (TABLE 1).[18] In rats and guinea pigs maintained on stock diets, the phospholipide P/N was unaltered. The relative specific activities of the phospholipides of the guinea pig are only slightly decreased from those of the rat. This difference becomes less when the relative radioactivity values of the guinea pig liver lipides are calculated for 1 gm. of fat-free tissue, which should represent the activity of a definite weight of tissue in synthesis phospholipides. We may conclude that the synthesis of phospholipides by the guinea pig is very similar to that of the rat, although a slightly decreased rate may be related to the absence of the hepatic choline oxidase.

The measure of phospholipide synthesis in the liver is a very primitive function and much stress has to be applied if there are to be alterations in this function. The harmful action of poisons, such as carbon tetrachloride and heavy metals, has long been known. Infections in different parts of the body, as well as parasitization of the liver cells themselves, alter liver functions.[19] The phospholipide synthesis in the plasma of untreated cirrhotic patients is very similar to that found in normal persons.[20] The phospholipide turnover in the liver of rats in experimental cirrhosis has been produced by carbon tetrachloride and has been found to be unaltered.[8, 21] The phospholipide turnover in the plasma of patients with infectious hepatitis, in which there is necrosis of the liver cells, has the same rate as normal individuals.[22]

The synthesis of liver phospholipide in animals maintained on various diets is constant when expressed as per gram of fat-free tissue.[23] Attempts to compare the rate of incorporation of P^{32} into the liver phospholipides and nucleoproteins of rats have been made (TABLE 2).[24] Male rats (100–110 gms.) were maintained 4–8 weeks on diets containing 25 per cent, 5 per cent Casein with respectively a low (5 per cent) or high (32 per cent) fat content. The rats were injected with P^{32} and, after 6 hours, the inorganic P, lipides, and nucleoprotein P and N were determined. From these results and the radioactivity measurements, the relative specific activity of the phospholipides and nucleoproteins were calculated. The liver phospholipides P/N was constant irrespective of the protein or fat content of the diet. The ratio of the relative specific activities of the phospholipide P/nucleoprotein P was unaltered by the protein

TABLE 2

AVERAGE VALUES OF PHOSPHOLIPIDE AND "NUCLEOPROTEIN" TURNOVER IN THE LIVER OF RATS ON VARIOUS DIETS

Diet					Phospholipide-P		Nucleo-protein-P		
					Relative specific activity				
Protein %	Fat %	Number of animals	Body weight gm.	Total lipides gm.	overall	per gm. of fat free tissue	Relative specific activity	Phospho-lipide-P/ Nitrogen	R.S.A. Phos-pholipide-P*/ R.S.A. Nucleo-protein-P
5	5	16	114	1.47	0.466	0.087	0.254	0.037	1.840
25	5	10	230	0.54	0.390	0.044	0.178	0.037	2.190
5	32	11	130	2.46	1.045	0.167	0.389	0.050	2.690

* R.S.A. = Relative specific activity. All animals maintained on the diet 8 weeks.

TABLE 3

AVERAGE VALUES FOR PHOSPHOLIPIDE AND "NUCLEOPROTEIN" TURNOVER IN THE LIVER OF RATS MAINTAINED ON A LOW PROTEIN-LOW FAT DIET*

				Phospholipide-P		Nucleo-protein-P		
				Relative specific activity				
Days on diet	Number of animals	Body weight gm.	Total lipides gm.	overall	per gm. of fat free tissue	Relative specific activity	Phospho-lipide-P/ nitrogen	R.S.A. Phospho-lipide-P†/ R.S.A. nucleo-protein-P
64	11	101	2.33	0.210	0.055	0.084	0.037	2.508
96	4	148	4.07	0.179	0.030	0.071	0.040	2.663
114	8	126	3.21	0.200	0.032	0.093	0.039	2.175
164	12	169	3.09	0.177	0.025	0.102	0.029	1.767
185	6	152	2.36	0.259	0.046	0.137	0.037	2.040
206	14	142	2.99	0.181	0.028	0.083	0.033	2.211

* 5% protein and 5% fat.
† R.S.A. = relative specific activity.

intake or by the amount of fat in the liver. Campbell and Kosterlitz[25] have shown that the phospholipide P content of a unit of liver cell is determined mainly by the dietary protein intake, a little by the fat intake, and not at all by the choline content of the diet. The ratio of phospholipide P/protein N, is constant over a wide range of dietary protein intake.[25]

Attempts to compare the rate of phospholipide and nucleoprotein synthesis in the production of experimental cirrhosis have been made (TABLE 3). In rats maintained on a 5 per cent Casein diet for 4, 8, 13, 16, and 28 weeks, the ratios of the synthesis of phospholipides to nucleoprotein and phospholipide P/N were constant and were not related to the degree of cirrhosis or fibrosis by histological evidence. There appears to be a constant relation of the synthesis of phospholipide to that of the nuceloprotein, whether or not the liver is "fatty," fibrosed, or normal.[24]

This constant rate of synthesis of liver phospholipide appears changed very

TABLE 4

EFFECT OF THIOUREA ON THE PHOSPHOLIPIDE TURNOVER IN THE LIVER OF RATS

Wks. on exp.	Substance given	No. of rats	Specific activity				
			Inorganic P	Lipide P		Lipide P × 100 / inorganic P	
					% change		% change
3	None	4	152	101		67	
	Thiouracil	4	157	79	−22	50	−25
	Thiouracil*	4	164	78	−23	47	−30
5	None	4	173	115		67	
	Thiouracil	4	187	96	−16	51	−24
	Thiouracil†	4	213	110	−4	52	−22

Rats fed a high protein diet (thiouracil 0.3%), then a low protein (thiouracil 0.6%) last week of experiment.
* Last day 100 mgm. thiouracil by stomach tube.
† Last day 100 mgm. thiourea by stomach tube.

TABLE 5

THE EFFECT OF THYROID GLAND ON THE PHOSPHOLIPIDE TURNOVER IN THE PLASMA OF MAN

Patient	Exp. conditions	Relative specific activities of plasma phospholipide		
		24 hours	48 hours	72 hours
R. B.	Thyrotoxicosis	0.731	1.588	1.85
	After surgery	0.292	0.805	1.03

little by dietary intake, but is influenced by metabolic requirements of the body. The thyroid gland plays a definite role in this metabolism.[26] In experimental animals, thyroxine increases the rate of synthesis both in plasma and liver.[27] Thiouracil and thiourea diminish the phospholipide turnover.[27, 28] As shown in TABLE 4, a significant decrease in the animals receiving the drugs is apparent. The plasma phospholipide turnover in a patient with clinical signs and laboratory finding of thyrotoxicosis showed a decreased rate of synthesis after partial thyroidectomy (TABLE 5).

Lipide phosphorylation is greatest in conditions in which a single dose of lipotropic agents are administered in the presence of a "fatty" liver. The effect of a dose of choline is chiefly due to an increased rate of formation of lecithins.[29] The stimulating effect of various incomplete methylated ethanolamine derivatives was greatest in the choline-containing fraction in rats maintained on a low protein diet.[30] In cirrhotic patients, with fatty infiltration of the liver by biopsy, a significant increase in the rate of phospholipide synthesis is usually demonstrable after a single dose of choline or methionine.[31]

The stimulating action of lipide phosphorylation in the liver has a low degree of structural specificity than other biological actions exerted by the same substance.[32] Thus, there are substances which, without being lipotropic, increase the turnover rate of liver phospholipides; *e.g.*, cystine,[33] ethanolamine,[34, 35] and diethanolamine.[15] In animals maintained on a low protein diet, the stimulat-

TABLE 6

THE EFFECT OF CORTISONE ON THE RATIO OF PHOSPHOLIPIDE AND "NUCLEOPROTEIN" TURNOVER IN THE RABBIT*

Tissue	Number of animals	Material given	Relative specific activity of phospholipide-P / Relative specific activity of nucleoprotein-P
Liver	10	—	1.32
	13	Cortisone	1.62
Kidney	4	—	1.94
	6	Cortisone	1.05
Lung	4	—	1.00
	7	Cortisone	1.15
Heart	4	—	0.62
	7	Cortisone	0.61
Skeletal muscle	4	—	0.69
	9	Cortisone	0.61

* Cortisone 7.5 mgm. I.M. daily for two months.

ing effect of ethanolamine was most pronounced in the noncholine-containing fraction of the liver lipides.[30] Diethanolamine presumably acts as a metabolic antagonist of the methyl acceptors in the synthesis of choline with the accumulation of an atypical cephalin.[15] This stimulatory effect of lipide phosphorylations by lipotropic agents is somewhat related to the previous diet, for it does not occur in rats on a stock diet[36] or in man in adequate protein intakes.[9, 10] Many other compounds have to be studied for their stimulating effect of lipide phosphorylations.[32] Cortisone, administered for two months in the rabbit, failed to change the rate of lipide phosphorylation in the liver (TABLE 6).[37] Apparently, the rate of synthesis of phospholipide by the liver cell is rather constant when related to a unit of tissue or nucleoprotein and is not influenced by the protein content of the diet, fat content of the liver, or degree of fibrosis. The stimulating effect of various lipotropic agents may represent a replacement of a deficiency of methyl donors or methyl acceptors,[12, 13] or merely a mass action effect.[35]

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ANTIBIOTICS AND LIVER INJURY*

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Dietary factors determining experimental injury in rats are summarized in TABLE 1.

The dietary factors beneficial in the prevention of cirrhosis as the more chronic form of hepatic injury may be identified by one common denominator; *i.e.*, by a sufficient supply of lipotropic factors, in particular of choline and its precursors. The benefit seen after administration of Vitamin B_{12} may be due to its choline-sparing effect. In more recent studies we found hog mucin effective as a lipotropic agent, and also in the prevention of dietary hepatic cirrhosis. Mucin is a good source of a new microbiological factor essential for the growth of a special strain of *Lactobacillus bifidus* var. Penn, and appears to be closely related to or identical with a similar factor found in human milk. Mucin appears to improve protein utilization.

The acute form of experimental dietary hepatic injury, acute massive necrosis of the liver, may be prevented by the sulfur-containing amino acids, cystine or methionine, or by vitamin E. In addition to these dietary factors, other dietary constituents such as Factor 3 of Schwarz, and to some extent fats rich in monoglycerides as well as some emulsifiers (for example, Tween 60) may delay the development of experimental dietary hepatic necrosis.

In contrast to cirrhosis, it is difficult to reconcile pure deficiency as the possible cause of dietary hepatic necrosis with the interchangeability of substances chemically as different as the sulfur-containing amino acids, cystine or methionine, and the fat-soluble vitamin E in the prevention of hepatic necrosis. The assumption has been made that the beneficial effect of the sulfur-containing amino acids and tocopherol more probably results from an underlying detoxifying mechanism than from the supply of a deficient nutritional factor. Both vitamin E and the sulfur-containing amino acids are known detoxifying agents which may counteract the noxious effect of hepatotoxic and related substances. In the case of dietary hepatic necrosis, such toxic substances may originate in the intermediary metabolism or under the influence of the intestinal flora, particularly in the large intestine.

Our first approach was directed toward the elimination—or at least modification—of the intestinal flora as a possible source of factors injurious to the liver. It has been shown that aureomycin, when added to the necrogenic experimental basal diet containing yeast as the sole source of protein, had a significantly beneficial effect in the prevention of experimental hepatic necrosis in rats. In contrast to vitamin E or the sulfur-containing amino acids, cystine or methionine, which as supplements to the basal experimental diet will permanently prevent the production of hepatic necrosis, aureomycin was found to delay as a rule the appearance of necrosis and thus its protective action was only temporary.

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* *Sponsored by the Commission on Liver Diseases, Armed Forces Epidemiological Board and supported in part by the Office of The Surgeon General, Department of the Army.*

TABLE 1
DIETARY FACTORS IN LIVER INJURY

	Cirrhosis	Necrosis
Protein	Beneficial	Beneficial
Methionine	Beneficial	Beneficial
Cystine	Injurious	Beneficial
Choline	Beneficial	No effect or injurious
Vitamin E	No effect	Beneficial
Dietary fat	Injurious	No effect or injurious
Vitamin B^{12}	Beneficial	No effect

In analyzing this effect of aureomycin, it should be pointed out that intensive studies have revealed no indication of an underlying systemic or focal hepatic infection as the direct cause of experimental dietary acute necrosis of the liver. Thus the choice lies among (1) the supply of a missing antinecrogenic substance; (2) a direct metabolic; and (3) an antimicrobial effect on the intestinal flora. As one possible "contaminant" we have used vitamin B_{12} as a supplement to the basal diet, with or without added aureomycin, and found it without any appreciable effect. Although a direct metabolic effect of aureomycin could not be excluded and it still remains a possibility, we favored interaction between aureomycin and the intestinal flora as the determining factor.

If the effect of aureomycin is mediated by the suppression of the intestinal flora, other antimicrobial agents should also prove to be effective, although not necessarily equal to aureomycin, depending mainly on their bacterial "spectrum" and on the ease with which they may produce resistant strains. Thus, it seemed advisable to study the effect of various antimicrobial agents on the production of dietary hepatic necrosis, especially in comparison with the effect of aureomycin.

In one of the experiments we compared aureomycin with polymyxin and streptomycin, and found polymyxin ineffective, streptomycin slightly effective, and aureomycin very significantly effective. The delay in the production of massive necrosis by aureomycin becomes particularly evident when the number of surviving animals is charted in relation to days of survival.

In addition to polymyxin, chloramphenicol and bacitracin were without effect on the production of dietary hepatic necrosis in rats. Sulfaguanidine had a slightly protective effective effect, whereas streptomycin, neomycin, and terramycin, in increasing order, were definitely effective.

Ingested streptomycin is absorbed from the intestinal tract only in traces. Thus its beneficial effect further suggests suppression of the intestinal flora, as the mode of action. Observations with penicillin are in good accord with this assumption. Whereas penicillin given by injection was either without any, or of only limited beneficial effect, penicillin given by mouth, especially its poorly soluble organic base salts, exerted very marked protection, not less than that of aureomycin.

With regard to this comparison between aureomycin and oral penicillin, the qualifying remark has to be made that, during the course of these investigations,

TABLE 2

Exp.	Date	Survival time in days (av.)	
		Control	Aureomycin
1	November 1949	34	110
2	January 1950	41	103
3	August 1950	34	58
4	October 1950	32	42
5	January 1951	27	34

the delaying effect of aureomycin on experimental hepatic necrosis became gradually less pronounced with successive experiments carried out during the last 1½ years. The results recorded permit the conclusion that the average survival time of rats kept under *identical* conditions and fed aureomycin in addition to the necrogenic diet has been considerably reduced, whereas in the same time the survival of the control rats (not receiving aureomycin) remained practically unchanged. See Table 2.

During this whole experimental period, the same strain of rat (Sprague-Dawley), with the same initial weight and with the same experimental diet, was used. Batches of aureomycin from 1949 were compared with more recent batches and the same reduction in the survival time of the treated rats was obtained with "old" and "new" aureomycin.

In January, 1951, when the aureomycin effect seemed to escape us, experiments with the identical strain of rats and the identical experimental diet (containing ingredients from a common source) were set up by us in two other laboratories, in which similar experiments had never been conducted previously. In both these new experiments, the original long and very significant delay by aureomycin in the development of hepatic necrosis was again observed. Further, the survival time of the control rats not receiving aureomycin was also significantly prolonged, compared with the figures given in the last table. These results appear to be in accord with the assumption that, in repeated experiments, (1) aureomycin-resistant bacterial strains and/or (2) an increase in virulence of an unknown agent, such as a virus, may have developed. Increase in aureomycin resistance has been demonstrated by us in rats receiving aureomycin-supplement for *Escherichia coli* and *Enterococcus*.

Since early 1951, more effort has been made to conduct the experiments under stricter hygienic conditions. Under these improved conditions and with a new batch of yeast as a constituent of the basal diet, a slight prolongation of the survival time for the control rats and very pronounced protection with neomycin, oral insoluble penicillin, aureomycin, and terramycin were observed.

The most impressive results were observed in preliminary experiments carried out in cooperation with Professor J. A. Reyniers and Doctor T. D. Luckey at the Germ Free Life Laboratory, University of Notre Dame. Following this presentation, Dr. Luckey will report on our observations in germ-free rats fed the necrogenic experimental yeast diet.

We also observed the effect of aureomycin, terramycin, and penicillin in the

TABLE 3

Treatment	Total liver fat (%)	Food intake (gm./day)
	25.4 ± 2.0	6.5 ± 0.2
Aureomycin	12.2 ± 2.2	6.1 ± 0.2
Terramycin	8.8 ± 0.7	6.7 ± 0.2
Methionine	13.2 ± 2.4	6.6 ± 0.2
Aureomycin Methionine	7.5 ± 0.7	6.0 ± 0.2
Terramycin Methionine	7.6 ± 0.9	6.0 ± 0.3

TABLE 4

No. of rats	Sex	Diet	Av. wgt. gain	Cirrhosis
10	M	Y5H	−41.2 ± 11.0	9
10	M	Y5H + A	+16.3 ± 6.5	0
10	F	Y5H	−34.8 ± 6.4	10
10	F	Y5H + A	+19.6 ± 3.6	2

production of fatty liver and dietary cirrhosis in rats fed a cirrhosis-producing ration low in protein (casein) and high in fat. In such rats, they exerted a very pronounced lipotropic effect. This effect may be noted on Table 3.

Aureomycin had a highly protective effect on dietary cirrhosis and its sequelae, such as ascites and renal injury. See Table 4.

Similar results were obtained with penicillin mixed with the food, either in the form of the free potassium or the slowly soluble organic basic salt. Chloromycetin was also effective when added to a cirrhosis-producing diet containing peanut meal and small amount of casein, as seen in Table 5.

The nutritional effect of antimicrobial agents when added to the necrogenic or cirrhosis-producing diet was not limited to the delayed appearance of hepatic necrosis or cirrhosis but it manifested itself also in promotion of growth. In the experiments dealing with hepatic necrosis, this gain in weight was obtained in varying degrees with all antimicrobial agents, irrespective of their effect on hepatic necrosis. Note Table 6.

During the last few years, the growth-promoting effect of antibiotics has been put to a successful practical test in animal husbandry and is generally considered to be mediated through the intestinal flora.

Although our original concept was centered around the toxic etiology of acute massive hepatic necrosis, the observations so far reported could also be explained by the elimination of bacteria from the intestinal tract which may use up protective constituents of the food ingested and, in consequence, reduce their supply for the body. Such mechanism may more easily explain the effect of antibiotics on the prevention of cirrhosis and growth than on the prevention of massive necrosis which, with the interchangeability of cystine and tocopherol as protective factors, makes the interaction of toxic factors, perhaps as a result of "intestinal autointoxication," a reasonable possibility to be strongly considered.

Sparing of an essential dietary constituent under influence of antibiotics has

TABLE 5

THE EFFECT OF ANTIBIOTICS ON THE DEVELOPMENT OF LIVER CIRRHOSIS IN RATS FED PEANUT MEAL DIET

Group	Supplement (mgm./daily)	Weight (gm.)		Cirrhosis		Kidney injury	
		Starting	Final	0	+	0	+
1	—	154 ± 2.7	252 ± 19.1	2	8	4	6
2	Aureomycin 5	154 ± 3.0	310 ± 7.8	8	2	8	2
3	Aureomycin 25	154 ± 2.7	307 ± 6.2	10		10	
4	Terramycin 5	154 ± 2.4	305 ± 6.1	8	2	8	2
5	Terramycin 25	152 ± 2.2	327 ± 4.6	10		10	
6	Penicillin 25	153 ± 1.8	307 ± 3.3	10		10	
7	Dibenz. Pen. 5	153 ± 2.1	325 ± 2.7	10		10	
8	Dibenz. Pen. 25	153 ± 2.3	320 ± 5.8	10		10	
9	Chloromycetin 5	152 ± 2.0	307 ± 10.0	7	3	9	1
10	Chloromycetin 25	152 ± 1.9	309 ± 5.5	10		10	

(Experimental period 150 days with 10 rats in each group)

TABLE 6

	Weight gain during first four weeks gm.
Controls	4.4 ± 1.3
Aureomycin	24.0 ± 1.05
Polymyxin	12.0 ± 1.05
Streptomycin	25.5 ± 0.9

been recently demonstrated by Popper and his associates on the example of choline. Two thirds of choline ingested is excreted by a normal person in the urine as trimethylamine or its oxidized form. Priming with aureomycin or penicillin will reduce the excretion of trimethylamine after ingestion of choline to very low levels. Intravenous injection of choline will not lead to urinary excretion of trimethylamine. These observations seem to indicate that the intestinal flora even in a normal person may convert a large proportion of ingested choline into trimethylamine. Suppression of the intestinal flora or, at least, of its choline-metabolizing constituents by aureomycin or penicillin will make more choline available for the body. By analogy, similar mechanisms may be postulated for other lipotropic food constituents, effective in the prevention of dietary cirrhosis.

Such easy solution, however, is not borne out by more intensive analysis of all participating factors. In the first place, in collaboration with Popper and his associates, we found that the sparing of dietary choline under the influence of aureomycin and penicillin is only a very temporary effect, lasting only for a few days. In spite of continuous medication with antibiotics, the original high excretion of trimethylamine re-establishes itself as observed before antibiotics were given. In view of the fact that cirrhosis prevention may last for 150–200 days, the short-lived sparing effect, at least as far as demonstrable, is not sufficient to explain the beneficial result which follows prolonged administration of antibiotics.

Further, in intensive studies, we were unable to demonstrate any permanent change in the composition of the intestinal flora as the result of antibiotic action. In these studies carried out in cooperation with Doctor Shockman, Doctor Forbes, and Miss Chu, temporary suppression of sensitive microorganisms is followed by the return of the original flora characteristic for the diet in question. The only remarkable and significant difference is the emergence of resistant strains. Thus, it is our present assumption that all the metabolic changes, such as prevention of hepatic injury or growth promotion, must be linked with changed metabolism of the microorganism comprising the symbiotic intestinal flora. There are indications that such changes may be reflected in the metabolism of the host organism as well, such as in the urinary composition. Our first effort was concentrated on the study of sulfur metabolism with special reference to fecal and urinary sulfur excretion. No difference could be found in the S-metabolism, or in the urinary excretion of gluconic acid, in rats fed the necrogenic yeast diet with or without antibiotics. The most promising results were obtained through the study of ether-soluble acids in the urine. In cooperation with Doctor Barness and Doctor Forbes, we found that rats fed the necrogenic diet supplemented with aureomycin or penicillin have a low total ESA excretion.

Beginning at approximately 30 days, the rats on a necrogenic diet, even when supplemented with vitamin E, excrete more ESA than do similarly fed rats on a necrogenic diet supplemented with penicillin or aureomycin.

On chromatographic analysis of the ether-soluble acids, we found four spots with RF values of 45, 78, 86, and 95. Spot 78 was identified as a mixture of isosuccinic acid and hippuric acid. Spot 95 is α,α-dimethylsuccinic acid. Spots 45 and 86 are as yet unidentified. α,α-Dimethylsuccinic acid makes up a greater proportion of the ESA in the aureomycin- and penicillin-treated animals. Spot 86 does not appear in the nonsupplemented animals or in animals receiving vitamin E. Spot 45 is of greater intensity in the supplemented than in the nonsupplemented or vitamin-E-treated animals.

The relation of these peculiar metabolic patterns to hepatic necrosis is under further study.

Discussion of the Paper

DOCTOR KLAUS SCHWARZ: It is a well-established fact that dietary necrotic liver degeneration is a deficiency disease that can be prevented by cystine, vitamin E, and Factor 3. Comparatively speaking, it is conjectural and more debatable whether "gastrointestinal intoxication" is causatively involved in the development of the disease. The argument of "gastrointestinal intoxication" has been used frequently in the medical literature to explain diseases the cause of which is unknown. At one time or the other, for example, gastrointestinal intoxication was held to be the reason of diseases such as hyperthyroidism and pernicious anemia.

That antibiotics such as aureomycin have a strong inhibitory influence on dietary necrotic liver degeneration is nothing unusual or special. It has been very clearly demonstrated in various laboratories that almost all vitamin B deficiencies in the rat can be inhibited or even prevented by the supplementation of various antibiotics[1, 2, 3, 4]. Their mode of action has not been clarified. I am sure, however, that it would be unjustified to infer from such observations that all these different manifestations of avitaminosis are "gastrointestinal intoxications."

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GERMFREE ANIMALS AND LIVER NECROSIS*

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During the latter part of 1949, discussions between the group working on the etiology of massive hemorrhagic necrosis of the liver at the University of Pennsylvania and the research staff at LOBUND Institute led to preliminary experiments early in 1950 to explore the possibility of using germfree rats in this study. It was found that the syndrome was readily produced in conventional rats from the LOBUND strain and that autoclaving the diet[1] did not materially alter its necrogenic character. Critical experimentation with germfree rats should determine whether the microbial flora affects the incidence of liver necrosis. The results obtained with germfree rats up to the present date are summarized in this report.

Methods

Germfree animals were reared and maintained in the Reyniers germfree system.[2] The germfree units are provided with locks in which materials such as food and water can be introduced into the cage with steam sterilization. Sterile air is obtained via glass fiber filtration. The operator cares for the animals through attached rubber gloves. The animals are housed in ordinary wire bottom rat cages or in metabolism cages when urine and fecal samples were collected. At weekly intervals, bacteriological examinations are made to establish whether the cage is germfree.[3]

The diet used was patterned after that of Himsworth.[1] The composition is: corn starch, 79.5 gm.; British brewer's yeast, 18.0 gm.; salts (USP III), 3.0 gm.; peanut oil, 5.83 gm.; cod liver oil, 1.17 gm.; thiamin ClHCl, 4.0 mg.; riboflavin, 0.4 mg.; pyridoxine Cl, 0.3 mg.; calcium pantothenate, 2.0 mg.; cobalamine, 0.02 mg.; and 2-me-napthaquinone, 0.2 mg. The diet is placed in a cloth bag in an inch-thick layer and treated in the sterile lock by exposure to a 25-in vacuum for 10 min. then free flowing steam for 5 min. followed by 25 min. steam sterilization at 17 p.s.i. A vacuum is then drawn for 10 min. and the food taken into the germfree cage: or taken out for the conventional animals. Food and water are presented *ad libitum.* The animals given vitamin E supplements are fed about 30 mg. α-tocopherol per os semiweekly.

In the first two experiments, Caesarean-born, hand-reared germfree rats were used; in the last two experiments, dam-suckled germfree animals were used. None of the differences found between germfree and conventional rats seemed to disqualify *a priori* the germfree rat as a proper test animal.

Results

The incidence of liver necrosis in the germfree and conventional rats is summarized in TABLE 1.

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* *Sponsored by the Commission on Liver Diseases, Armed Forces Epidemiological Board and supported in part by the Office of The Surgeon General, Department of the Army and the Office of Naval Research.*

TABLE 1

EFFECT OF NECROGENIC DIET ON GERMFREE AND CONVENTIONAL RATS

Status	Supplt. to diet	Av. initial wt. in gm.	Growth gm./day	Days on diet at death	No. of rats dead with liver necrosis*/no. started
Germfree	None	85	1.2	74 & 79	0/2
Conventional	None	84 ± 5	0.7	165 ± 97	5/6†
Germfree	None	40	1.4	39 (34 & 43	0/2
Conventional	None	42 ± 3	0.7	33 ± 3	8/8
Germfree	None	42	1.9	69	0/2
Germfree	Vit. E	42	1.8	69	0/2
Conventional	None	47 ± 4	0.5	41 ± 11	8/8
Conventional	Vit. E	54 ± 4	0.9	180	
Germfree	None	39	1.7	91 & 145	0/2
Germfree	Vit. E	37	1.6	145	0/2
Conventional	None	46 ± 5	0.8	68 + 23	12/12
Conventional	Vit. E	42 ± 4	1.0	150	0/4

* Incidence of necrosis based on histological examination by Doctor Harry Goldblatt.
† One died with lung abscess.

In the first experiment, rats with an initial weight of 85 gm. were started on the necrogenic diet. The germfree rats survived for some 74 and 79 days on diet, and their livers were found to be normal. The control rats, however, did not come down with necrosis until much later, rendering the experiment inconclusive. The cause of death of the germfree animals was found to be associated with disturbances of the blood-clotting mechanism as evidenced by internal hemorrhages with a distribution typical of a vitamin K deficiency and delayed clotting (capillary method) and prothrombin times[4] (TABLE 2).

In the second experiment, younger rats were used. Eight conventional control animals died with liver necrosis in an average time of 32 days. The germfree animals survived for 34 and 43 days. One germfree animal had an elongated tooth and did not seem to eat well. It was found moribund on the 34th day and the animal was killed. Necropsy revealed hemorrhagic areas in the lungs. The other animal was found to be moribund on the 43rd day. The animal was bled from the abdominal aorta, and clotting time and prothrombin time were both found to be delayed compared to that of conventional rats. The livers of both rats were found to be normal.

Since vitamin E was the only vitamin lacking which was thought to be required during this period, the third experiment included two germfree rats supplemented with vitamin E and two without supplement. The eight conventional controls promptly came down with necrosis in an average of 41 days. The germfree rats ate more and grew somewhat better than the conventional animals. This experiment was discontinued at 69 days due to accidental contamination. No abnormal pathology was observed, and the livers were all normal. Vitamin E supplement did not alter the weight gain of germfree animals.

TABLE 2

ALTERATIONS IN BLOOD CLOTTING AND HEMOLYSIS OF RED BLOOD CELLS IN GERMFREE AND CONVENTIONAL RATS

Status	Supplement	Days on diet	Clotting Time (Min.)		Hemolysis[5] after	
			Whole blood	Prothrombin[4]	10 hrs.	20 hrs.
Germfree	None	34	*			
	None	43	38	60		
	None	74	*	10		
	None	79	*			
	None	91	*		—	—
	None	145	80	60	—	—
	Vit. E	145	12	5.9		
	Vit. E	145	12	6.4		
Conventional	None	27			+†	+†
	None	68	0.5	0.5	+	+
	None	68	0.5	0.3		
	None	70	0.5	0.3		
	Vit. E	113	0.5	0.3	—	—
	Vit. E	113	0.5	0.3	—	—

* Clotting times were not taken in these animals; however, extensive hemorrhages found at death in lungs and other organs. Their livers were normal.

† 8 rats (all other data are expressed for individual rats).

In repeating the third experiment, 12 conventional control rats died with liver necrosis in an average time of 68 days. No hemorrhages were evident in the two germfree rats supplemented with vitamin E. The two unsupplemented germfree animals lived 91 and 145 days, respectively, and had no liver necrosis. One animal died at 91 days after a blood sample was taken from the tail and on necropsy was found to have a stomach hemorrhage. The other rat showed no pathology.

The vitamin E stores of the unsupplemented germfree rats were estimated by the hemolysis test of Rose and György.[5] The negative hemolysis observed for these rats (TABLE 2) would indicate that the rats were not depleted of their stores of vitamin E.

Discussion

Thus far, we have been unable to induce liver necrosis in germfree rats using a diet which regularly produced lethal liver necrosis in conventional rats.

The observed disturbances in the clotting mechanism of the germfree rats were suggestive of a deficiency of vitamin K. In our experience, a vitamin K deficiency is not easily induced in germfree rats. Although 40 per cent of the vitamin K may be destroyed by autoclaving as a dietary constituent,[3] the quantity remaining should be ample to satisfy any requirement if it is properly absorbed. Hove has reported lung hemorrhages in vitamin E deficiency.[6]

In the hemolysis test, red blood cells from rats deficient in vitamin E hemolyze readily in the presence of dialuric acid. Thus, the erythrocytes of conventional rats fed the necrogenic diet normally hemolyze after 1–6 hours exposure to dialuric acid. In simultaneous tests, erythrocytes of nonsupplemented germfree rats failed to hemolyze even after 20 hrs. Such a negative hemolysis reaction is seen only in conventional animals receiving vitamin E and thus could be indicative of a high vitamin E reserve in the germfree animals. Since alterations in blood-clotting mechanism were partially remedied by the supplementation of vitamin E (TABLE 2) the nonsupplemented animals did not necessarily have full stores of vitamin E after 3–5 months on the diet.

Summary

In a series of four experiments, eight germfree rats were found to have normal livers at autopsy. All but one of the 34 conventional rats, fed the same diet, died with massive hemorrhagic necrosis of the liver. The germfree rats exhibited prolonged clotting time which was partially prevented by vitamin E supplementation.*

Acknowledgments

This work was supported, in part, by contract with the Office of Naval Research and the Army Medical Corps. The authors acknowledge, with thanks, the help of Dr. H. A. Gordon, Chief Pathologist; Morris Wagner, Chief Bacteriologist; B. A. Teah, Supervisor of Germfree Production and J. Pleasants, biochemist from LOBUND Institute, and Dr. Harry Goldblatt of the Institute for Medical Research, Cedars of Lebanon Hospital, Los Angeles.

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* Definite hemorrhagic necrosis of the liver was observed in five germfree rats since this report was given. The germfree rats reported herein ate almost twice as much as conventional rats, whereas the germfree rats which developed liver necrosis were fed a diet restricted in quantity to that of the conventional control rats.

HEPATIC INJURY DUE TO CONDITIONED SULFO AMINO ACID DEFICIENCY*

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Functional and structural changes resulting from nutritional deficiency of amino acids and, specifically, of sulfo amino acids are well known. This paper will present examples of lesions resulting from drainage of sulfo amino acids from the body or from biological antagonism to them. Since these functional and structural changes can be prevented or corrected by additional administration of relatively large amounts of the respective amino acids, they are considered examples of conditioned amino acid deficiency.

Bromobenzene combined with cysteine is excreted in the urine after acetylation. This represents a mechanism of depletion of cysteine and its precursors such as methionine from the liver and growth inhibition after administration of bromobenzene has been described.[1, 2] If 150 mgm. of bromobenzene is given intraperitoneally to a fasting rat, an extensive centrolobular necrosis develops within 48 hours. It is characterized by eosinophilic necrosis, hydropic swelling and, finally, disappearance of the liver cells associated with interstitial infiltration by inflammatory cells[3] (A in FIGURE 1). The lesion is more advanced in instances in which fasting for more than 12 hours precedes the bromobenzene injection and massive necrosis with hemorrhage imitating nutritional hepatic necrosis may be noted. Administration of cysteine or methionine in large doses prevents the development of the lesion (B in FIGURE 1). This already suggests the possibility that drainage of sulfo amino acids, rather than an intoxication with the halogenated benzene, causes the lesion. Chemical analyses confirm the histologic findings.[4] In comparison to controls, the phosphatase activity of the liver tissue is increased and the esterase and succinic dehydrogenase are significantly reduced in rats acutely intoxicated by bromobenzene. Similarly, serum bilirubin is elevated, and bromsulfalein retention is noted. In the animals treated simultaneously with methionine and cysteine, most of these changes are either entirely prevented or at least reduced (FIGURE 2).

Additional evidence supports this concept. The mercapturic acid excretion resulting from the administration of bromobenzene is markedly increased by the simultaneous administration of cysteine and methionine resulting in prevention of the hepatic lesion (TABLE 1). The greater portion of bromobenzene is detoxified by means other than mercapturic acid excretion such as formation of glucuronates or sulfates. Nevertheless, only a small increase of the fraction of bromobenzene detoxified as mercapturic acid is associated with the protection. This suggests that the protection is not due to the removal of relatively small amounts of bromobenzene by detoxification via mercapturic acid but rather is due to repletion or sparing of the sulfo amino acids in the hepatic pool. In the case of glycine used for hippuric acid formation, such a pool of 10

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* Supported by a grant from the Doctor Jerome D. Solomon Memorial Research Foundation.

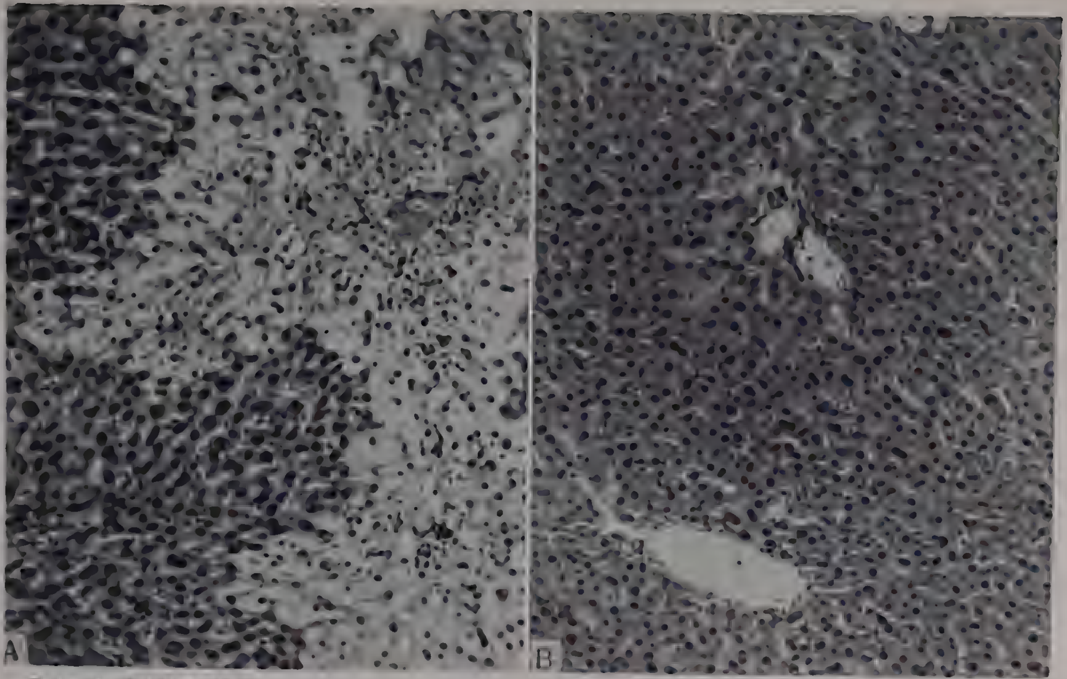

FIGURE 1. Rat [illegible]. A. (×120) 48 hours after administration of bromobenzene. Extensive central necrosis with hydropic swelling of the liver cells and inflammatory infiltration is seen. B. (×120) 48 hours after administration of bromobenzene and methionine. The architecture is not distorted.

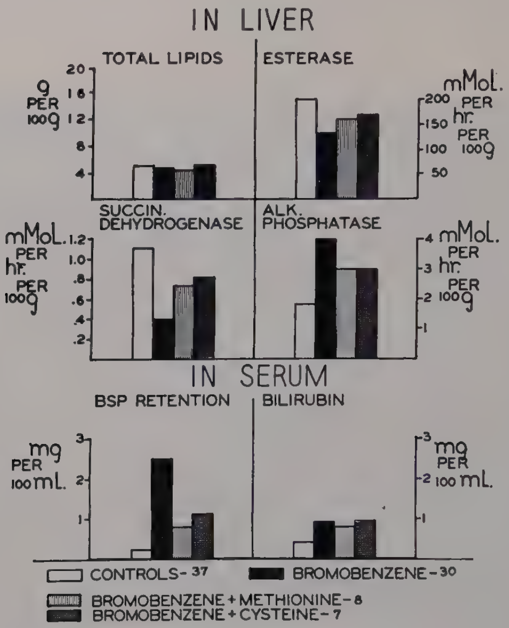

Figure 2. Results of chemical determinations on liver tissue and serum of control rats and rats sacrificed 48 hours after administration of 150 mgm. bromobenzene/100 gm. body weight with or without supplemental administration of 300 mgm. methionine or cysteine/100 gm. body weight. (Small figures in the legend indicate the number of animals.)

Table 1

Urinary Mercapturic Acid Excretion of Control Rats and of Animals Which Had Received 48 Hours before 150 mgm. Bromobenzene per 100 Gm. Body Weight with or without Additional Administration of 300 Gm. Methionine or 300 Gm. Cysteine/100 Gm. Body Weight

	Mecapturic acid	
	As mgm. cysteine	As % bromobenzene injected
Controls	1	—
Bromobenzene	32	13.9
Bromobenzene + Methionine	53	23.2
Bromobenzene + Cysteine	52	23.1

mg./100 gm. body weight has been demonstrated.[5] In the case of bromobenzene, 30 mgm. cysteine are made available for mercapturic acid formation.

The cysteine binding takes place on the para position of the bromobenzene molecule. Consequently, benzenes dihalogenated in the para position such as para-dibromobenzene and para-chlorobromobenzene do not produce the lesion. In contrast, meta-dichlorobromobenzene with the para position free produces it (table 2). This is confirmed by low mercapturic acid production with the para compounds and high production with the meta compound. Again, the

TABLE 2

URINARY MERCAPTURIC ACID EXCRETION OF RATS DURING 48 HOURS FOLLOWING THE ADMINISTRATION OF EQUIVALENT AMOUNTS OF BROMOBENZENE OR DIHALOGENATED BENZENES

	Mgm./100 gm. body weight	Mercapturic acid	
		As mg m. cysteine	As % halogenated compound injected
Bromobenzene	75	18	15.6
m-chlorobromobenzene	91	17	16.5
p-chlorobromobenzene	91	5	4.3
p-dibromobenzene	112	4	3.5

percentage of the halogenated compound excreted as mercapturic acid is rather small. These findings militate strongly against a primary toxicity of the halogenated benzenes, but support drainage of sulfo amino acids as the cause for the hepatic necrosis. Finally, in animals in which spontaneous liver damage occurred as reflected in focal necroses apparently due to infection, bromobenzene injection failed to produce the characteristic necrosis. If the concept presented is correct, the process of detoxification as such may become detrimental to the liver.

Another example of conditioned amino acid deficiency in the sense described above is the effect of ethionine, an amino acid similar to methionine except that the terminal methyl group is replaced by an ethyl group. This supposed biological antagonist to methionine[6, 7] has been shown to produce pancreatic necrosis followed by pancreatic fibrosis and atrophy[8, 9, 10, 11] and dilatation of the pancreatic ducts upon prolonged treatment. In late stages the acinar cells reveal an irregular regeneration. Whereas in early stages the islands appear entirely normal, hydropic swelling of the island cells results in some of the animals treated for longer than one month (FIGURE 3). In some of these animals the urine contains reducing substances. However, the blood sugar curve is normal and further investigations are required to elucidate this glycosuria. These lesions in an organ as sensitive to protein deficiency as the pancreas are prevented or corrected by methionine administration.

Intraperitoneal administration of single doses of ethionine produces fatty liver in female rats but not in males.[7] Females are protected by administration of testosterone and males are made sensitive by castration.[12] This points to a protective effect of male sex hormone as far as the acute fatty liver is concerned. It can best be explained by a protein-sparing effect of testosterone preventing the fatty liver, yet insufficient to inhibit the pancreatic degeneration. Again, administration of methionine prevents the lesion. The histologic alterations in acute ethionine intoxication are chemically reflected in bromsulfalein retention and marked elevation in liver lipids, but enzyme changes in the liver otherwise found in hepatic injury are absent[13, 14] (FIGURE 4).

Upon administration of a diet containing approximately 0.4 per cent methionine in its protein and supplemented with 0.5 per cent ethionine, a diffuse fatty

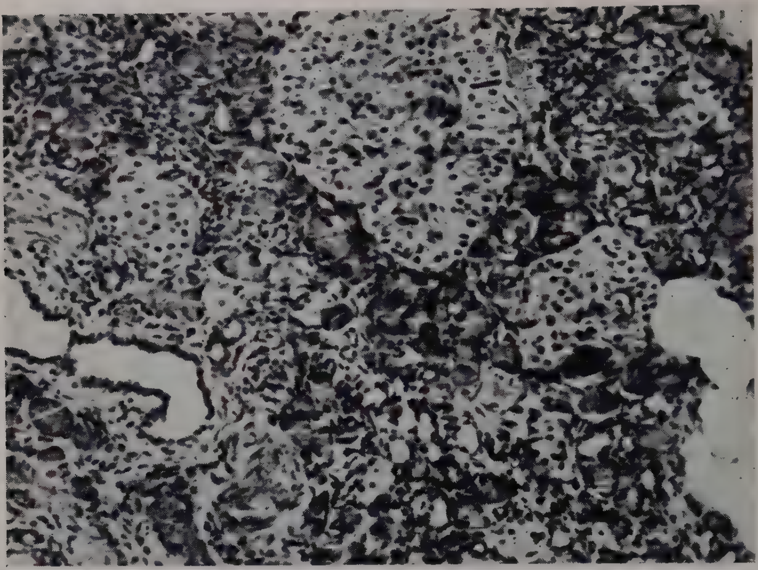

FIGURE 3. (×150) Pancreas of rat which had been on 0.5 per cent ethionine diet for 51 days. There is hydropic swelling of the island cells, distortion of the acini with marked regeneration of the acinar epithelium, dilatation of the ducts, and interstitial inflammatory infiltration.

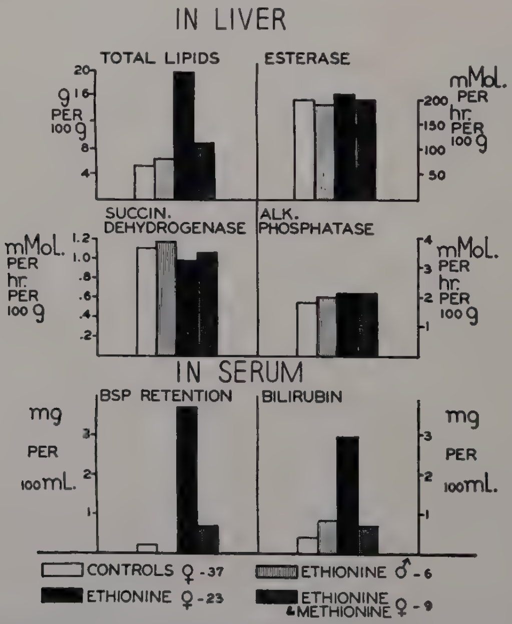

FIGURE 4. Results of chemical determinations on liver tissue and serum in control rats as well as in rats which had received 100 mgm. ethionine per 100 gm. body weight 48 hours before sacrificing, with or without supplemental administration of 300 mgm. methionine per 100 gm. body weight. (Small figures in the legend indicate the number of animals.)

liver develops within approximately one week. After about 12 days, fat is seen only in the central lobular zone. However, now there is central necrosis and streamers of interstitial infiltration extending into the lobular parenchyma chiefly around intralobular cholangioles (A in FIGURE 5). After three weeks, extensive interstitial infiltration around the cholangioles is associated with marked liver damage and, after four weeks on the diet, a diffuse hepatic lesion is noted in male as well as in female rats.[15] It is characterized by diffuse hepatocellular damage with hemogenization of the cytoplasm recognized by reddish discoloration in Mallory's aniline blue or Gomori's trichrome-stained section (B in FIGURE 5). This damage often progresses to cytolysis and is also associated with bizarre regeneration of the liver cells which reveal large nuclei and large and often multiple nucleoli (A in FIGURE 6). Moreover, there is a dense interstitial infiltration by reticuloendothelial cells. In the center of the lobules, lipofuscin deposition reflects preceding central necrosis. The fibers of the reticulum framework reveal extensive proliferation. However, dissection of the lobules by septa is only occasionally seen. There is increased visibility of intralobular cholangioles, the presence of which in the normal liver was recently emphasized by Elias.[16] These intralobular cholangioles are not easily seen but become readily apparent if the common duct is ligated. The septal and interlobular bile ducts proliferate and the latter may reveal marked dilatation (B in FIGURE 6). This is also associated with a marked dilatation of the large extrahepatic biliary duct to about 2 mm. in diameter, similar to the changes recently described in mice bearing grafted pituitary tumors.[17]

The characteristic lesion described is associated with reduced fat and protein content; low esterase and high phosphatase activity in hepatic tissue; and increased bilirubin, markedly high alkaline phosphatase, and low esterase activity in the serum. The morphologic and chemical alterations can be prevented if one per cent methionine is added to the diet or when the animals are returned for one week or longer to the stock diet (FIGURE 7). During the course of the chronic ethionine intoxication the hepatic lipids first show a peak and subsequently a depression below the normal level whereas the proteins consistently decrease (FIGURE 8).

The characteristic diffuse hepatocellular degeneration with cholangiolitis and interstitial infiltration is subject to various influences. Only few rats survive the simultaneous administration of cortisone and ethionine, since most of them die during this period from focal hepatic necrosis apparently due to infection and not due to ethionine. However, rats which survive the simultaneous administration have nearly normal livers. Administration of large doses of choline with the diet prevents the inflammatory changes but produces marked regenerative changes of the liver cells. The hepatic lesion caused by chronic ethionine feeding is not significantly altered by administration of pancreatin, vitamin B_{12}, penicillin, or a high-fat or low-protein diet. It is markedly aggrevated by pregnancy, or guanidoacetic acid which acts as a methyl depletor, or menthol which acts as a glycogen depletor.

Rats only occasionally survive on the 0.5 per cent ethionine diet for longer than five months. In the rats which do or in others in which periods of ethionine administration are alternated with stock diets or, finally, in rats which have

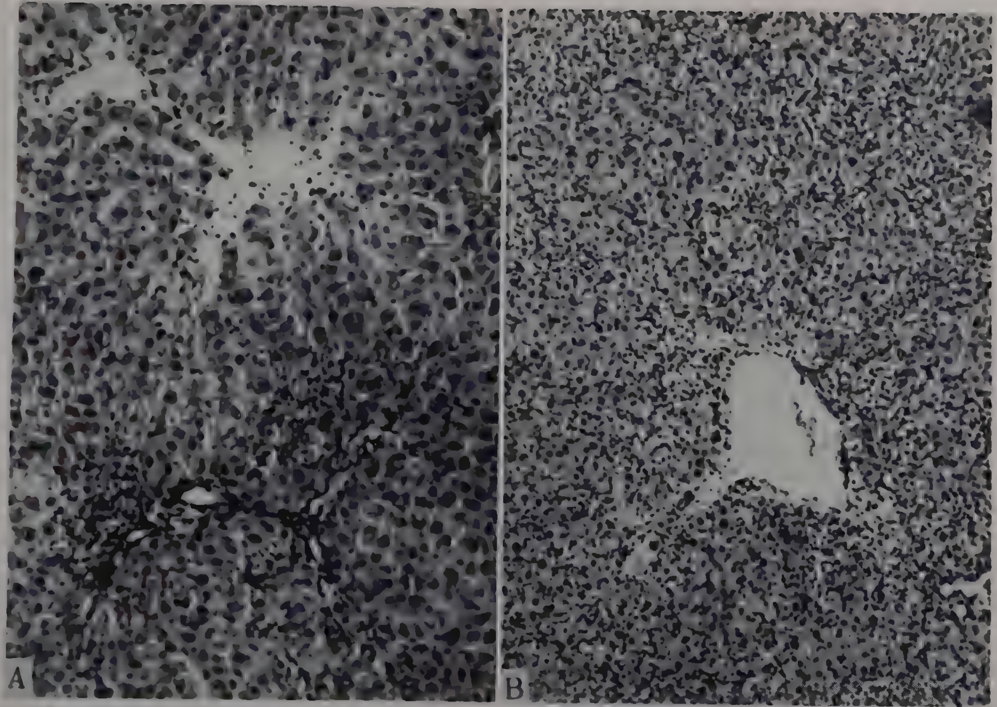

FIGURE 5. Rat livers. A. (×105) After 17 days on the 0.5 per cent ethionine diet. Small central necroses are seen. Interstitial inflammatory infiltration extends from the portal triads along cholangioles which are visible in increased amount. B. (×65) After 35 days on the 0.5 per cent ethionine diet. Extensive interstitial infiltration obscures the lobular pattern. The liver cell plates are irregularly arranged and bile ducts and cholangioles are proliferated.

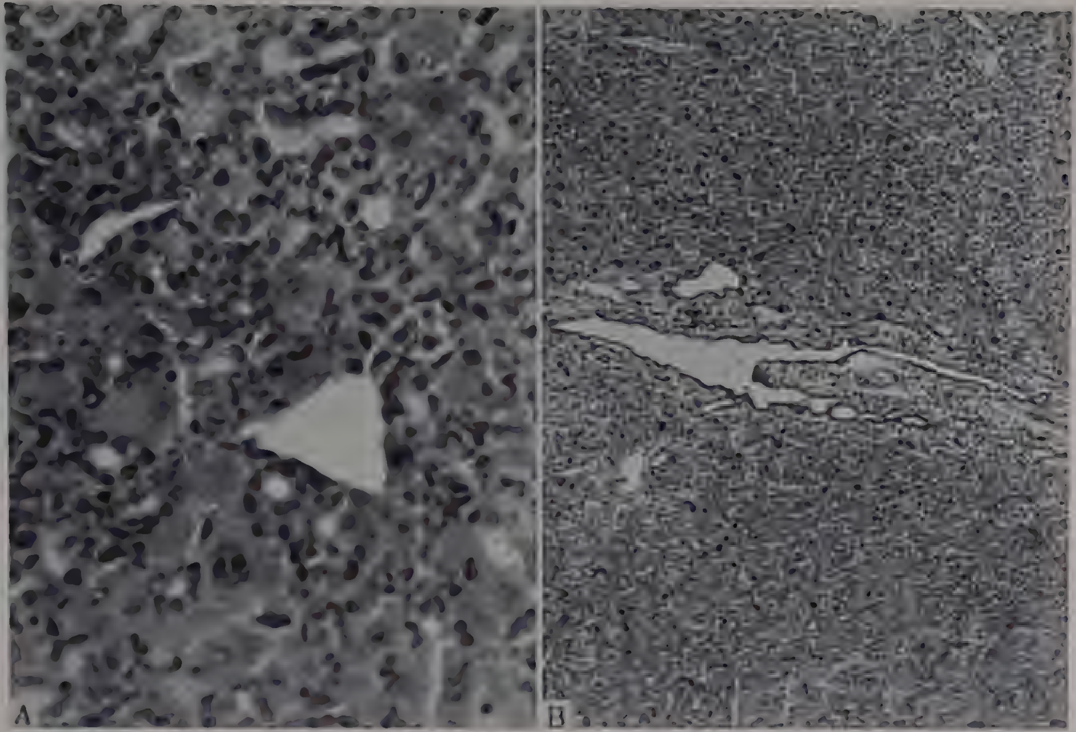

FIGURE 5. Rat livers. A. (×265) After 51 days on the 0.5 per cent ethionine diet. The liver cells show cytolysis as well as irregular regeneration. The nucleoli are large. The cholangioles are proliferated. There is extensive interstitial infiltration by round cells. B. (×66) After 51 days on the 0.5 per cent ethionine diet. Marked dilatation of larger bile ducts is noted.

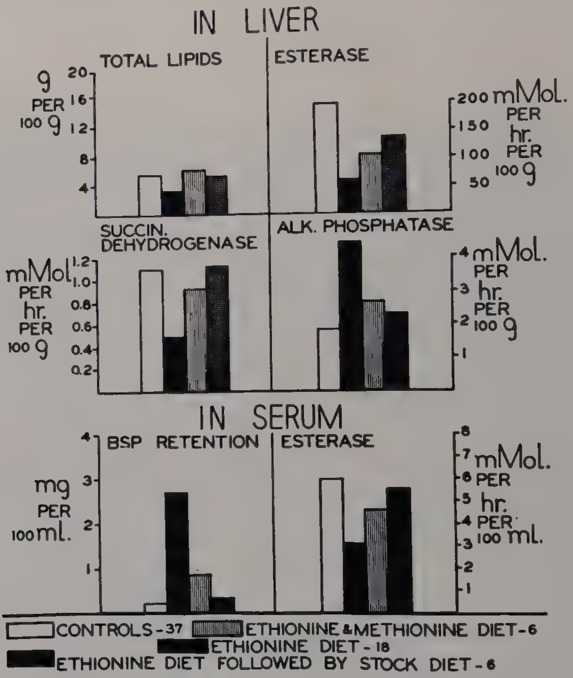

FIGURE 7. Result of chemical determinations on liver tissue and serum in control rats as well as in rats which have been on the 0.5 per cent ethionine diet for 28 days with or without supplemental 1 per cent methionine and in rats which have been on the ethionine diet for the same period and subsequently on the stock diet for 10 days. (Small figures in the legend indicate the number of animals.)

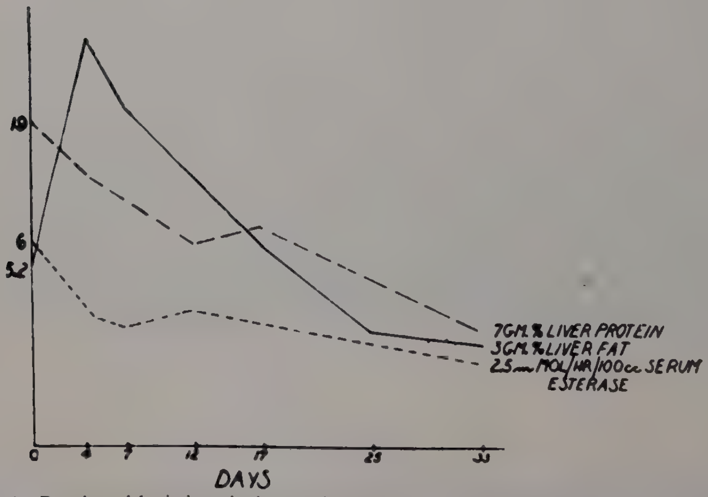

FIGURE 8. Protein and fat in hepatic tissue and esterase in serum in rats for various periods on 0.5% ethionine diet.

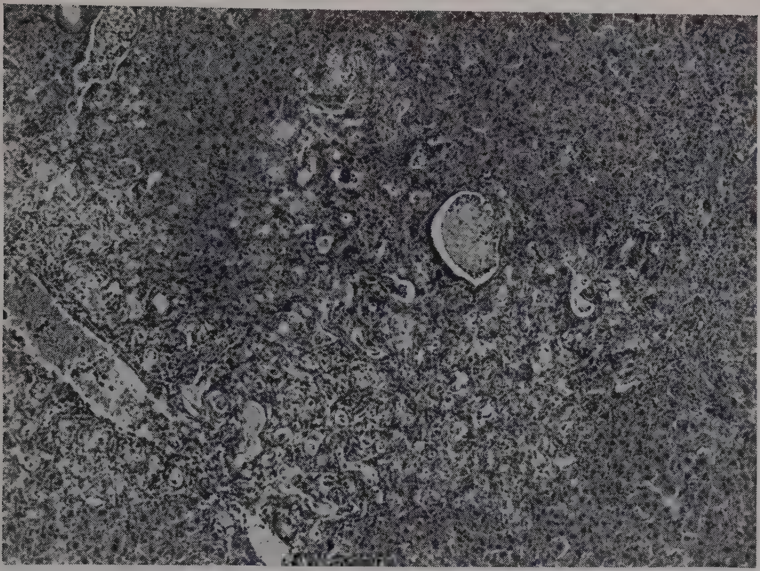

FIGURE 9. (×65) Liver of rat on prolonged feeding with 0.5 per cent ethionine. Cholangio-fibrosis composed of proliferated and dilated bile ducts filled by mucoid material and lined by epithelium revealing irregular mitoses.

been kept for approximately 100 days on diets containing 0.2 per cent ethionine, the lesions are markedly intensified. The bile duct epithelium may reveal bizarre and excessive proliferation characterized by irregular mitoses and by piling up on part of the wall of the duct. Eventually, multiple lesions develop which are arranged around portal triads. They may form nodules in which the ductal lumen is dilated, the lining is almost papillomatous, and the content gives a mucus reaction (FIGURE 9). The lesions simulate the cholangio-fibrosis or cholangiomas produced by carcinogenic azo dyes. Similarly, the liver cells reveal excessive regeneration charaterized by groups of cells which are arranged in several-cell-thick plates instead of one-cell-thick plates. This is clearly seen in reticulum fiber stains in which groups of cells are surrounded by fibers in contrast to the extensive fiber proliferation between single-cell plates in the surrounding liver tissue (A in FIGURE 10). These groups of cells may become larger. In some of the specimens, grossly visible nodules up to 2 mm. in diameter are noted, in which the liver cells are different from the surrounding tissue in staining qualities of nuclei and cytoplasm. Also the marked inflammatory and fibrotic reaction as well as the cholangiolar proliferation in the surrounding tissue is completely absent (B in FIGURE 10). As with the nodular bile duct proliferations, no information is as yet available as to the biological behavior of these liver cell nodules. Nevertheless, their histologic characteristics strongly suggest them to be hepatomas, at least of the benign variety. Further study is required of these bile duct and liver cell nodules. Usually, more than one such lesion has been found in the 17 rats with nodules. Their response to methionine treatment particularly requires study.

Thus, the ethionine lesion imitates the picture of human malnutrition, especially kwashiorkor, since it starts with fatty metamorphosis, proceeds to

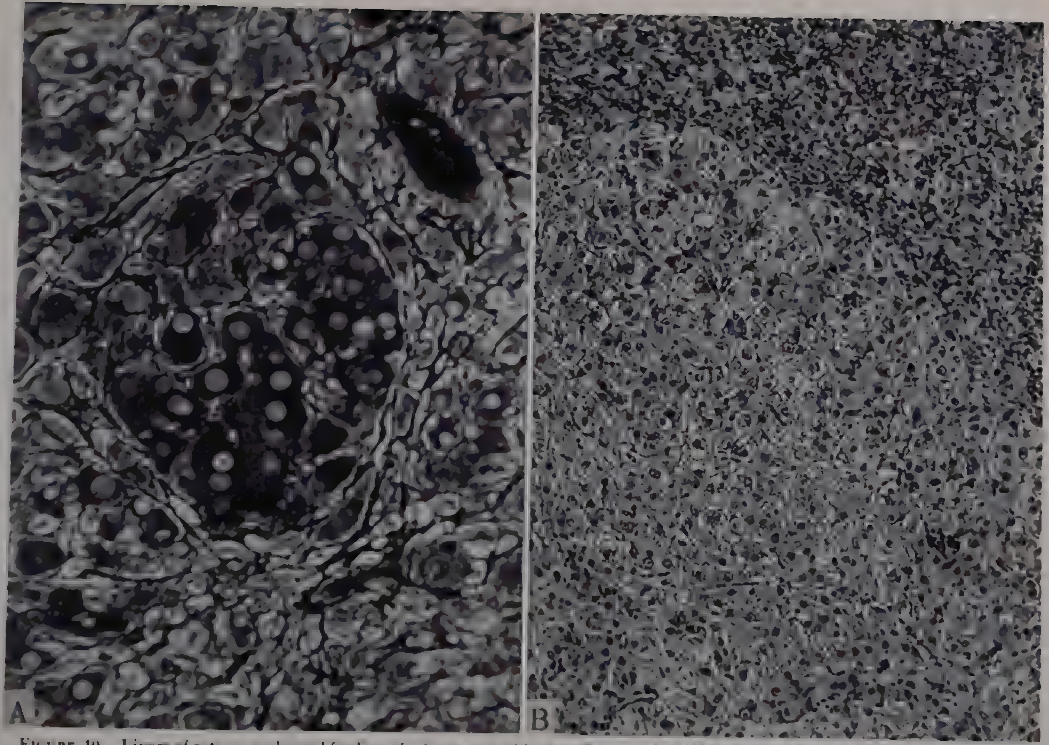

FIGURE 10. Livers of rats on prolonged feeding of 0.5 per cent ethionine diet. A. (×240) Reticulum fiber stain. Markedly proliferating liver cells are arranged in multiple layers without intervening fibers and form small nodules which differ from the surrounding tissue in which extensive reticulum fiber proliferation is seen. B. (×55) Hematoxylin-eosin stain. Partial view of a nodule (2 mm in diameter) composed of liver cells arranged in several-cell thick plates and revealing large nuclei with large nucleoli. The appearance of the cells differs from that in the surrounding tissue, exhibiting extensive interstitial infiltration, diffuse fibrosis, and irregular arrangement of the liver plates.

fibrotic changes, and terminates in lesions at least suspicious for hepatic tumor formation, and since it is associated with pancreatic alterations. An amino acid antagonism has to be assumed rather than a toxic effect of ethionine in producing these lesions, although the latter cannot be completely excluded. Triethyl choline, a possible metabolite of ethionine,[18] does not produce hepatic and pancreatic alterations under similar conditions.

It thus appears that most of the available evidence militates against a toxic effect of both bromobenzene and ethionine and suggests that depletion of, or antagonism to, sulfo amino acids is the basis of the lesions demonstrated, in which toxicity and deficiency meet. The lesions represent patterns of a conditioned amino acid deficiency in a broad sense of "malnutrition caused by conditions other than a primary dietary deficiency".[19] It may be worth while to search for a similar pattern in man which might explain lesions which are due not to nutritional deficiency but to internal interference with amino acid and protein metabolism.

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CLINICAL EVALUATION OF A HIGH-PROTEIN, HIGH-CARBOHYDRATE, RESTRICTED-FAT DIET IN THE TREATMENT OF VIRAL HEPATITIS*†

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Introduction

The relationship between diet and certain types of liver injury has been established in the case of animals,[1, 2, 3, 4, 5] and specific liver-protective factors have been described.[4, 6, 7] Results of such studies, however, do not appear to be directly applicable to viral hepatitis in man because this disease has not been transmitted successfully to animals. The clinical importance of diet in the treatment of hepatitis in man has often been emphasized, but the degree of this has not been entirely clear. The quantity of fat in the diet has been thought by some to be a factor,[8, 9, 10, 11, 12] while a number of other workers have considered that the amount of protein in the diet was of importance.[7, 10, 13 14, 15, 16] The absence of any clear indication of the effectiveness of any particular type of diet arises, in part, from the problems encountered in conducting clinical nutrition studies. These include difficulties of experimental design in what amounts to a clinical trial involving a number of variables,[17, 18] the prolonged nature of such studies in hepatitis, the lack of early specific diagnoses in many situations,[15] and the absence of established uniform methods of evaluation.

The present study was directed towards the evaluation of the effect of a high-protein diet on the course of serum hepatitis in a group of volunteer subjects experimentally inoculated with materials containing the agent(s) of viral hepatitis. The opportunity to study these patients was afforded in the course of a program concerned with the sterilization of blood and blood products with respect to serum hepatitis which is being carried out by the National Institutes of Health under the auspices of the Commission on Liver Disease, Armed Forces Epidemiological Board, and with the cooperation of the Bureau of Prisons, U. S. Department of Justice. The findings reported here are the product of study of 67 controlled cases of induced homologous serum hepatitis which occurred among volunteers in two institutions.

Conduct of Study

The volunteer participants were men between the ages of 21 and 35. In the latter part of the study, men of 30 years and over were not accepted. Final selection was based upon a personal interview, a medical history, a physical examination, and a review of the available records to determine the suitability of the volunteers on the basis of age, physical condition, mental status, and personality. In addition, complete series of liver function tests were run including serum bilirubin (one-minute and total), thymol turbidity, cephalin flocculation, thymol flocculation, total lipids, zinc turbidity, and urine urobilinogen and bilirubin.

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*This investigation was conducted, in part, under contract from the Office of the Surgeon General, Department of the Army, and under the sponsorship of the Commission on Liver Disease, Armed Forces Epidemiological Board. It was made possible through the cooperation of the Bureau of Prisons, U. S. Department of Justice, and of the staffs of the U. S. Penitentiaries at Lewisburg, Pa., and McNeil Island, Wash.

† This study was initiated by J. W. Oliphant, M.D., deceased.

The nature of the over-all project made it possible to observe the course of the disease from the earliest appearance of abnormal laboratory or clinical findings. It was possible, therefore, to study the effects of diet starting at a much earlier period in the illness than could normally be possible in the case of casual hospital patients. Every effort was made to take advantage of this feature of the study by placing patients on a dietary regimen as early as possible in the course of their illness. Good control was achieved and the design was such as to provide for the collection of data in terms of well-defined periods in the patient's course from pre-inoculation to discharge.

Throughout the entire study, two diets applicable or adaptable for clinical use were employed. As the subjects manifested signs or symptoms of hepatitis, they were admitted to the hospital and placed on a high protein, high carbohydrate, restricted fat diet, referred to here as the "special" diet, or on an "ad lib." diet.

The "special" diet was well-balanced, interesting, and palatable, as well as being simple to prepare, measure, and control. It provided at least 200 gm. of protein, 75 gm. of fat, and 680 gm. of carbohydrate, approximating 4000 calories per man per day. Between-meal feedings were avoided to obtain maximum voluntary food consumption.

The "ad lib." diet consisted of the same food provided for the general institution population. There were no restrictions made on the type, character, quantity, or quality of food other than the general limitations of the institution diet.

Subjects remained on the respective diets from the time of admission until discharged from the hospital, except where medical emergencies developed.

The food supplied both diet groups was handled in exactly the same manner. All servings including nutrient liquids were individually weighed or measured

TABLE 1

	"Special" diet				"Ad lib." diet				Difference			
Period	Cal.	Protein	Fat	CHO	Cal.	Protein	Fat	CHO	Cal.	Protein	Fat	CHO
Acute	3616	203.3	78.8	541.5	2213	77.5	78.2	307.3	1403.0	125.8	0.6	234.2
Recovery	3954	221.0	80.1	599.0	2833	102.6	98.0	392.0	1121.0	118.4	18.1	206.9
Total	3844	215.2	79.2	579.4	2609	93.8	90.5	361.7	1235.0	121.4	14.5	217.7

The following standard references were used:

United States Department of Agriculture Bulletin No. 8, Composition of Foods, June 1950. Bulletin No. 8 is a revision of the United States Department of Agriculture Publication No. 572, developed in cooperation with and approved by the National Research Council's Food and Nutrition Board.

Food Values of Portions Commonly Used, Bowes and Church. Seventh Edition, 1951. This publication is used for special items of food combinations. This publication is currently used by the Nutrition Branch, Preventive Medicine Division, Office of the Surgeon General, United States Army, and the Department of Agriculture, Division of Human Nutrition and Home Economics.

TABLE 2
DIETARY VALUES IN GRAMS

Sampling period (days)*	Diet	By calculation		By chemical analysis	
		Protein	Fat	Protein	Fat
7	Special	210.5	86.3	192.3	68.6
7	Ad lib.	111.3	110.7	94.9	96.9
7	Special	209.3	87.8	198.1	76.7
7	Ad lib.	101.6	100.9	92.9	77.6
7	Special	215.0	97.0	193.0	100.7
7	Ad lib.	106.5	106.9	100.5	76.0
7	Special	203.7	93.9	212.1	80.2
7	Ad lib.	100.2	110.1	92.8	76.3
7	Special	254.9	116.1	219.2	117.0
7	Ad lib.	100.8	107.1	94.3	81.5
7	Special	241.4	122.1	209.6	97.7
7	Ad lib.	105.4	119.3	97.5	87.0
7	Special	221.3	84.3	221.8	90.6
7	Ad lib.	113.3	106.0	91.6	95.9
7	Special	208.0	93.1	190.9	67.9
7	Ad lib.	127.6	138.8	96.2	96.8
5	Special	171.8	68.3	229.0	91.1
5	Ad lib.	104.3	101.3	96.7	75.2
5	Special	190.5	70.9	226.6	85.3
5	Ad lib.	118.3	96.9	133.1	73.9
1	Special	253.3	61.0	288.6	105.2
1	Ad lib.	116.8	104.8	104.6	78.0
2	Special	229.8	110.2	223.2	103.7
2	Ad lib.	99.2	95.9	86.3	81.3
2	Special	221.3	72.0	311.1	61.7
2	Ad lib.	133.9	141.2	139.8	103.0

* Complete meals were homogenized in a blender. Aliquots of each meal were pooled for the period indicated and samples of these pools set aside for chemical analysis.

under supervision. Edible waste or uneaten food was also weighed and the amount recorded. Food intake figures were calculated for each patient using these data. Sample meals representative of both diets were chemically analyzed for comparison with the calculated values.*

Sixty-seven volunteers who developed hepatitis with jaundice were included in the present study. Of these, 32 were in the "special" diet group and 35 in the "ad lib." diet group. During the time this study was being conducted, a total of 85 individuals was started on one or the other of the dietary regimens.

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* Chemical analyses were performed by Doctor Cyrus E. French, Professor of Animal Nutrition, College of Agriculture, Pennsylvania State College and through Captain E. B. Adams of the Sixth Army Medical Laboratory, Fort Lewis, Wash.

A number of these patients were already in the hospital with hepatitis when the dietary study was commenced. Since only those cases in which it was possible to calculate dietary intakes for the complete period of hospitalization were considered for inclusion in the group, eleven of these initial subjects were excluded, seven on the "special" diet and four on the "ad lib." diet. Two further cases, both on the "special" diet, were excluded because of uncertainty as to their dietary intake,* as were five individuals for whom a diagnosis of hepatitis with jaundice could not be sustained. All of these 18 individuals were eliminated before the statistical analysis was made. Intially, the distribution of patients between the two diet groups was on a one-for-one basis; a separate series of alternations being followed in each of the two institutions. Due to the imblance of the numbers between the two diet groups occurring as a result of the elimination of the above 18 individuals (five on the "ad lib." diet and 13 on the "special" diet), the last ten patients admitted to the dietary study were distributed on a two-for-one basis.

The cases of heptitis studied resulted from parenteral inoculation of the following infecting materials:

A. *Infected pool plasma.* A single lot of infected plasma was used in all the work relating to the safety of blood products. This pool was prepared from commercially procured plasma to which had been added some 800 ml. of plasma containing the "Fort Bragg" strain of homologous serum hepatitis virus as well as a total of approximately 600 ml. of plasma or serum from four reputed cases of serum hepatitis. The original volume of the material was approximately 130 liters, and it is referred to here as "infected pool plasma." The subjects who received this material represent a homogeneous group.

B. *Infected pool plasma, treated.* This represents materials which remained infective after treatment by one of the following processes:

(1) Ultraviolet irradiation at various levels;
(2) Heating for 2 hours at 60° C.;
(3) Heating for 4 hours at 60° C.;
(4) Dilution to 10^{-3} and 10^{-4};
(5) Treatment of β-propiolactone at different levels; or
(6) Storage at room temperature for 3–6 months.

Subjects who received these materials represent a heterogeneous group in the sense that, although they all received infected pool plasma, this had been treated in a variety of ways, each presumably affecting the virus in different degree.

C. *Carrier material.* Six suspected carriers of homologous serum hepatitis were examined by inoculation of their plasma into volunteer subjects. Five of the six were proved to be carriers. In addition, a few cases resulting from first-passage infected pool material have been placed in this group.

Cases resulting from the inoculation of some of the "carrier materials" had short incubation periods in the range 30 to 70 days, whereas those who received infected pool plasma had incubation periods between 60 and 120 days. This difference in incubation periods, coupled with a somewhat different clinical picture in which the severity of symptoms at the onset of illness was greater in relation to the laboratory findings than would be expected on the basis of our experience with the other infecting materials studied, suggests that some of the cases receiving "carrier material" may have had infectious hepatitis induced by parenteral inoculation.

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* One of these subjects received infected pool plasma, the other infected pool plasma, treated. The periods during which their total serum bilirubin levels remained above 1.0 mgm. per 100 ml. were 63 days and 49 days respectively.

D. *Thrombin.* Thrombin from a commercial batch reported to have caused cases of hepatitis in clinical use was inoculated into ten subjects in order to determine the safety of thrombin with respect to homologous serum hepatitis. Four of the subjects who developed hepatitis were studied from the dietary point of view. They do not fall into any of the above groups, so it is difficult to evaluate them except from the over-all point of view as to the development of complications.

The distribution of the 67 cases between the two diet groups, broken down by type of infecting material is shown at the foot of TABLE 3A.

Each patient's course was divided into the following periods:

(1) The pre-inoculation period during which the volunteer was being screened for the study.

(2) The incubation period, beginning with inoculation and extending to the time at which a diagnosis of hepatitis could be made.

(3) The period of illness which was divided into the acute period and the recovery period.

These periods were defined roughly by changes in liver function tests and, therefore, permitted objective comparisons.

Cases of all degrees of severity were encountered, including three which ended fatally. These latter cases had been respectively inoculated with infected pool plasma; infected pool plasma, treated; and carrier material.

TABLE 3A
DURATION OF ILLNESS IN DAYS—TSB 1.0 TO 1.0

Infected pool plasma		Infected pool plasma treated		Carrier material		Thrombin	
Spec.	Ad lib.	Spec.	Ad lib.	Spec.	Ad lib.	Spec.	Ad lib.
79	78	36	70	63	46	22	25
62	86	49	56	81	49		13
85	86	63	130	92	35		27
98	102	42	84	21	28		
93	70	56	49	30	43		
*	47	28	56	74	24		
102	45	53	47	*	47		
105	45	15	46	56			
94	63	45	18				
	50	*	70				
		15	67				
		35	21				
		14	42				
		98	22				
			59				
Total No. Cases...9	10	14	15	8	7	1	3

* Fatal cases (not included in the analysis).

The statistical evaluation of the effect of the two diets was made in terms of the duration of illness of the 67 cases. It should be noted that in such a comparison when recovery is the end point, it is not feasible to include cases which do not recover. For this reason, the three fatal cases are excluded from this analysis, although it is obvious that they must be taken into account when evaluating the results.

Discussion of Statistical Methods

Most of the analysis was based on measures of duration such as the number of days during which liver function tests, particularly the total serum bilirubin (TSB), remained abnormal or the number of days of clinical illness, *etc.* The data have been examined in a number of ways to try and discern differences between the cases on the "special" diet and those on the "ad lib." diet, and

TABLE 3B

DURATION OF ILLNESS—TSB 1.0 TO 1.0

Diet	Infected pool plasma	Infected pool plasma treated	Carrier	Thrombin	Sum
Special x_1	718	549	417	22	1706
$\bar{x}_1$	89.7500	42.2308	59.5714	22.0000	58.8276
n_1	8	13	7	1	29
Ad lib. x_2	672	837	272	65	1846
$\bar{x}_2$	67.2000	55.8000	38.8571	21.6667	52.7429
n_2	10	15	7	3	35
Sum X	1390	1386	689	87	3552
$\bar{x}$	77.2222	49.5000	49.2143	21.7500	55.5000
N	18	28	14	4	64
$d_i = \bar{x}_1 = \bar{x}_2 =$	22.5500	−13.5692	20.7143	0.3333	
$w_i = \frac{n_1 n_2}{N} =$	4.4444	6.9643	3.5000	0.7500	15.6587
$w_i d_i =$	100.2212	−94.5000	72.5946	0.2500	78.5658
$w_i d_i^2 =$	2259.9881	1281.2894	1505.7064	0.0833	5048.0672

$$(\Sigma w_i w_d)^2/\Sigma w_i = 394.1952$$

ANALYSIS OF VARIANCE

Source	Sum of squares	df	Mean square	Mean square	df	Sum of squares	Source
Diet ignoring material	587	1			3	14607	Material ignoring diet
Interaction	4654	3	1551.29	1551.29	3	4654	Interaction
Material	14414	3	4804.67	394	1	394	Diet
Between cells	19655	7					
Within cells	27455	56	490.27				
Total	47110	63	Interaction $F = \frac{1551.29}{490.27} = 3.16$ $F_{05} = 2.78$				
Mean	197136	1					

TABLE 3C

DURATION OF ILLNESS—TSB 1.0 TO 1.0

Source	SS	df	MS	
		Infected Pool Plasma		
Mean	107339	1		$F = \frac{2260}{321} = 7.04 \quad F_{05} = 4.49$
Total	7397	17		
Diet	2260	1	2260	
Within diet	5136	16	321	
		Infected pool plasma, treated		
Mean	68607	1		$F = \frac{1282}{673} = 1.90 \quad F_{05} = 4.22$
Total	18789	27		
Diet	1282	1	1282	
Within diet	17507	26	673	
		Carrier		
Mean	33909	1		$F = \frac{1501}{391} = 3.84 \quad F_{05} = 4.75$
Total	6189	13		
Diet	1501	1	1501	
Within diet	4697	12	391	

TABLE 3D

DURATION OF ILLNESS—TSB 1.0 TO 1.0

Source	SS	df	MS	
		Special Diet		
Mean	100360	1		$F = 8.76 \quad F_{05} = 2.99$
Total	24558	28		
Material	12590	3	4197	
Residual	11968	25	479	
		Ad lib. diet		
Mean	97363	1		$F = 4.32 \quad F_{05} = 2.91$
Total	21965	34		
Material	6477	3	2159	
Residual	15488	31	500	

when differences existed, to determine their statistical significance. A "five per cent" level of significance was decided upon before any calculations were undertaken. This implies that a difference, measured in terms of its standard error, could have occurred by chance only once in 20 times.

Tables 3A, 3B, 3C, and 3D illustrate the analysis in the case of the duration of illness as judged by the time the total serum bilirubin remained above 1.0 mgm. per 100 ml.

Three fundamentally separate tests have been employed in this analysis: the tests for duration, body weight change, and the incubation period.

It was apparent early in the analysis that the variation in the measures selected for study was influenced by the type of infecting material as well as by diet. The analysis of the data relating to the period before diets were started brings out a number of differences associated with the type of infecting material. One such characteristic is the incubation period. The average incubation periods were: infected pool plasma, 81 days; infected pool plasma, treated, 103 days; and carrier material, 61 days.

Another characteristic of the differences between infecting materials which is free of diet effect is body weight loss during the incubation period. The respective average differences are: infected pool plasma, −5.4 lbs.; infected pool plasma, treated, −5.5 lbs.; and carrier material, −3.6 lbs.

The weight loss data are based on fewer than the 67 patients, because either the inoculation or hospitalization weight was not obtained in a few instances. However, if the assumption is accepted that a missed weight could occur with the same probability in the group receiving different infecting materials, then the available numbers are sufficient to establish the difference between infecting materials.

The data on incubation periods and on loss of body weight occurring during the incubation period can be used to test whether bias was introduced in the assignment of patients to the diets. If bias existed, it might result in a preponderance of patients with short incubation periods being assigned to one of the diet groups. No such difference was found. Similarly, there was no difference between diets as measured by weight loss during the incubation period.

Results

A. *Food consumption levels.* TABLE 1 summarizes the calculated food intake levels in terms of average consumption during the acute period, the recovery period, and for the total period of illness. It will be seen that differences of well over 1,000 calories, 100 gm. in the case of protein, and over 200 gm. in the case of carbohydrate, were maintained between the two diets.

B. *Chemical analysis of diets.* Chemical analyses of the diets were made to confirm calculated values. These are compared in TABLE 2.

C. *Effect of diet.* In the course of the analysis, significant interaction between diet and the different infecting materials was found; that is, the effect of diet was not consistent with respect to infecting material. Where this interaction appeared in the analysis, it was necessary to analyze separately the effect of diet within each group which received different infecting material. The following measures of effect were used:

(a) The number of days the TSB remained abnormal when 1.0 was taken as the endpoint (duration of time the TSB was elevated above 1.0 mgm. per 100 ml.).

(b) The number of days during which the bilirubin remained abnormal when 1.5 mgm. per 100 ml. was taken as the endpoint. As a corollary to this, the period of time taken for the TSB to fall from 1.5 to 1.0 was studied.

(c) The number of days for the TSB to reach its maximum level.

(d) The number of days for the TSB to return form maximum to 1.0 mgm. per 100 ml.

(e) The number of days during which the patient was ill; *i.e.*, from the time of onset of illness until the patient could be said to be improving clinically.

(f) The duration of the recovery period; *i.e.*, from time of the onset of clinical recovery until the bilirubin reached 1.0.

(g) Weight change during illness.

(h) Average maximum TSB levels in each of the diet groups. This measure of effect can also be used in part as a test for bias.

The above measures of effect have been analyzed statistically. It must be emphasized that (a) through (g) are interrelated and do not constitute separate analyses. They represent measures of the duration of illness or portions of it as judged by different end points.

The results are summarized in TABLE 4. They reveal the following:

(1) With return to a bilirubin level of 1.0 mgm. per 100 ml. taken as the

TABLE 4

EFFECT OF DIET

Measure of effect	Infecting material	Average		Remarks
		Special diet	Ad lib. diet	
		Days†		
Duration of illness (TSB 1.0 to 1.0)	IPP*	90	67	*Significant* diet effect
	IPP, treated	42	56	No significant diet effect
	"Carrier"	60	39	No significant diet effect
Duration of illness (TSB 1.0 to 1.5)	IPP	72	52	No significant diet effect
	IPP, treated	24	36	No significant diet effect
	"Carrier"	45	23	*Significant* diet effect
Duration from TSB 1.5 to 1.0	All groups	17	16	No significant diet effect
Duration from TSB 1.0 to max.	All groups	20	16	No significant diet effect
Duration from max. TSB to 1.0	All groups	39	37	No significant diet effect
Acute period	All groups	14	17	No significant diet effect
Recovery period	IPP	55	47	No significant diet effect
	IPP, treated	25	35	No significant diet effect
	"Carrier"	41	21	No significant diet effect
Incubation period	All groups	85	88	No significant difference between diet groups
		mgm./100 ml.		
Maximum TSB	All groups	11.0	9.7	No significant difference between diet groups
		lbs.		
Wt. change between inoculation & TSB 1.5	IPP	1.4	−8.6	*Significant* diet effect
	IPP, treated	−2.1	−6.3	*Significant* diet effect
	"Carrier"	−1.0	1.1	No significant diet effect

* Infected pool plasma. † To nearest whole day.

endpoint of recovery:

(a) Subjects who received infected pool plasma and were given the "special" diet had a course of illness which was on the average 23 days longer than the corresponding individuals on the "ad lib." diet. This difference was statistically significant.

(b) Subjects who received infected pool plasma, treated, and were given the "special" diet had a course of illness which was on the average 14 days shorter than corresponding individuals on the "ad lib." diet. This difference did not reach statistical significance.

(c) Subjects who received "carrier material" and were given the "special" diet had a course of illness which was on the average 21 days longer than corresponding individuals on the "ad lib." diet. This difference was not statistically significant.

(2) With return to a bilirubin level of 1.5 mgm. per 100 ml. taken as the point of recovery, results were similar and in the same direction in each of the categories listed in 1(a), (b), and (c) above. The differences, however, reached statistical significance only in the case of "carrier material." When the period between the 1.5 TSB and 1.0 TSB levels was studied, no statistically significant effect of diet could be demonstrated.

(3) In calculating the average duration of time between a bilirubin level of 1.0 and the maximum TSB, no statistically significant diet effect was demonstrable.

(4) In calculating the average duration of time between the maximum TSB and the endpoint of recovery at 1.0, no significant diet effect was demonstrable.

(5) In calculating the average duration of the acute period, no statistically significant diet effects were apparent. Because of interaction, however, it was necessary to make separate calculations with respect to infecting materials when examining the average recovery periods. Here the differences were analogous to those obtained in 1(a), (b), and (c). However, the differences were not statistically significant.

(6) There were no significant diet effects on weight change during the period from hospitalization to the fall of TSB to 1.5.

D. *Effect of infecting materials.* The statistical evaluation disclosed a marked effect due to the type of infecting material used. Whenever interaction was present, it was necessary to carry out the analysis with respect to each of the measures of effect within each diet group separately. These results are summarized in TABLE 5. Reference to this table discloses the following:

(1) Within each diet group, there was a significant effect due to the infecting material used, whether the endpoint of the duration of illness was measured by the return of the TSB level to 1.5 or to 1.0.

(2) There was a significant effect of the infecting material on the period between a TSB level of 1.5 and a TSB level of 1.0.

(3) There was no significant effect of infecting material on the average number of days from 1.0 to the maximum TSB level. There was, however, a significant effect on the average number of days from the maximum TSB level to

TABLE 5

EFFECT OF INFECTING MATERIAL

Measure of effect	Diet	Average			Remarks
		Infected pool plasma	Infected pool plasma treated	Carrier	
			*Days**		
Duration of illness (TSB 1.0 to 1.0)	"Special"	90	42	60	*Significant* effect
	"Ad lib."	67	56	39	*Significant* effect
Duration of illness (TSB 1.0 to 1.5)	"Special"	72	24	45	*Significant* effect
	"Ad lib."	52	36	23	*Significant* effect
Duration from TSB 1.5 to 1.0	Both	16	19	15	*Significant* effect
Duration from TSB 1.0 to max.	Both	24	15	17	No significant effect
Duration from max. TSB to 1.0	Both	53	34	33	*Significant* effect
Acute period	Both	17.5	12	18	No significant effect
Recovery period	"Special"	55.5	25	41	*Significant* effect
	"Ad lib."	47	35	21	*Significant* effect
Incubation period	Both	81	103	61	*Significant* effect
			mgm./100 ml.		
Maximum TSB	Both	15.4	8.0	10.1	*Significant* effect
			lbs.		
Wt. change between Inoc. & TSB 1.5	"Special"	1.4	−2.1	−1.0	No significant effect
	"Ad lib."	−8.6	−6.3	1.1	*Significant* effect

* To nearest whole day.

1.0. The same effects were apparent when the period of illness was broken down into acute and recovery phases.

(4) There was a significant effect of infecting material on the average maximum TSB levels.

(5) There were significant differences between the mean incubation periods of the infecting materials used.

(6) There was no significant effect on weight changes of infecting material during the period between hospitalization and fall of TSB to 1.5.

E. *Relation between diet and the development of complications.* All cases on the dietary study were evaluated with respect to development of the following complications: death, coma, pre-coma, recrudescence, relapse, prolonged illness (more than 4 months), and residua persisting beyond the time of discharge from the program. The latter included persistent hepatomegaly, splenomegaly, right upper quadrant pain or tenderness, or failure of hepatic tests to resume pre-inoculation levels. Since these complications cannot be given equal

TABLE 6

Definitions	Special no.	Diet %*	Ad lib. no.	Diet %*
A. Comatose and fatal cases	4	12.5	0	0
B. Cases as above + those having pre-coma	5	15.6	1	2.9
C. Cases as above + those with recrudescence	13	40.6	5	14.3
D. Cases as above + those with relapse	15	46.9	6	17.1
E. Cases as above + those with prolonged illness	15	46.9	7	20.0
F. Cases as above + those with residua	18	56.2	8	22.8

All but one of the above definitions, (B), yield statistically significant differences indicating a higher occurrence of complications among those who received the special diet.

* In terms of the total number cases in each diet group.

weight, a number of definitions employing increasingly restrictive criteria were applied. The definitions and the number of cases falling into each are shown in TABLE 6.

The definitions listed are to some extent arbitrary and as they are not independent it must not be assumed that the data have been subjected to six different tests, one corresponding to each definition. The figures presented in the table indicate a more frequent occurrence of complications among the 32 patients on the "special" diet than among the 35 patients on the "ad lib." diet.

In compiling this table, cases were counted only once. Cases with more than one of the listed complications were counted only under the most restricted definition. Two of the cases listed under definition A require special mention. These subjects were members of a group of five volunteers who had been inoculated with plasma obtained from a proved carrier who had developed hepatitis with jaundice some 6 months before the plasma was collected. These two cases developed fulminating hepatitis with incubation periods of 44 and 46 days. One of the cases terminated fatally. The remaining three subjects in the group did not develop hepatitis. The unusual severity of the illness produced by this particular inoculum suggests, though this cannot be proved, that the outcome was related more to the infecting material than to the diet. Even if the two cases are removed from the table, however, the over-all results are not appreciably changed.

Discussion

All of the cases of hepatitis included in the present study were of so-called "inoculation hepatitis." Most were undoubtedly serum hepatitis, but there is some reason for believing that the illness resulting from the inoculation of some of the "carrier materials" may have been infectious hepatitis. Because of this, the broader term "viral hepatitis" has been used in the title.

The present cases developed in the course of a study on the sterilization of blood and blood products. This study was limited in scope, and there was no way of knowing beforehand how many cases of hepatitis could be anticipated. Consequently, no effort was made to randomize patients with respect to both diet and infecting material. A simple one-for-one allocation of patients between the diet groups was followed, in the hope that a practical randomization would thereby occur. A study of the incubation periods of patients who received different inoculating materials (a measure free of the effect of diet) indicates that this objective was indeed achieved.

The special feature of the present study is the fact that it was possible to place patients on one or other of the dietary regimens at the earliest appearance of signs, symptoms, or laboratory findings suggestive of hepatitis. This early institution of dietary therapy must be taken into consideration in any interpretation of the results of the present study.

The severity and duration of illness in viral hepatitis are extremely varied. In the present study, the analysis was based only on objective findings such as the duration of elevation of the total serum bilirubin, and it should again be emphasized that, although different endpoints and subdivisions of this measure of effect were used in the analysis, these are not independent but are in effect parts of the same analysis.

Summary

(1) A group of 67 volunteers taking part in studies on viral hepatitis has been evaluated with respect to two diets: a high-protein, high-carbohydrate, restricted-fat diet and an "ad lib." diet.

(2) It was possible to institute the diet in each case at an early point in the illness.

(3) The type of infecting material had a significant effect on most of the measures of duration studied.

(4) Apparent differences in effect of the two diets did not reach the level of significance with most of the measures of duration studied. In those instances where this did occur, the average duration of illness of patients on the "ad lib." diet was shorter.

(5) The occurrence of complications was more frequent among patients receiving the high-protein, high-carbohydrate, restricted-fat diet.

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